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<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of South Florida Tampa, Florida 33620  E-Mail: <a href="mailto:ccontrol@moffitt.usf.edu">ccontrol@moffitt.usf.edu</a> #			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
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<b>13. ABSTRACT (Maximum 200 Words)</b>  The goals of the Advanced Cancer Detection Center include the discovery of molecular and genetic markers of cancer risk, the identification of individuals at high risk for cancer through screening and the testing of methods to prevent cancer. The projects included in this report are: <ul style="list-style-type: none"><li>• Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers (PI: Tockman)</li><li>• The Specific Role of Isoflavones in Reducing Hormonal and Proliferative Risk Parameters in Prostate Cancer (PI: Kumar)</li><li>• Development of the Moffitt Cancer Network (PI: Krischer)</li><li>• African American Families with Inherited Breast or Ovarian Cancer (PI: Sutphen)</li><li>• Molecular Fingerprint of STAT3 Regulated Genes for Early Detection of Human Cancer (PI: Jove)</li><li>• The Tampa Bay Ovarian Cancer Study (PI: Sutphen)</li><li>• Molecular Predictors of Disease Behavior in Thyroid Cancer (PI: Muro-Cacho)</li><li>• Significance of Bax-dependent Apoptosis in Prevention and Detection of Human Prostate and Lung Cancer (PI: Dou)</li></ul> Each of these projects is presented as a complete study in the attached materials.				
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## **INTRODUCTION:**

The **Advanced Cancer Detection Center (ACDC)** of the H. Lee Moffitt Cancer Center and Research Institute at the University of South Florida received initial funding in October 1997. The creation of the Center followed a proposal that was developed in response to legislative language accompanying an appropriation in the Department of Defense budget that appeared in the September 28, 1996, Congressional Record:

“\$3,500,000 is available only for the establishment of an advanced cancer detection center for military personnel, dependents, and retired service members, using a network that is in close geographic proximity and includes the following: a military hospital, a regional TRICARE provider, a Department of Veterans Affairs hospital or hospitals, and a medical facility with a focused cancer center that meets the National Cancer Institute eligibility requirements, with respect to research funding. The conferees would expect this center to conduct coordinated screening for cancer detection and treatment, to train military cancer specialists, and to develop improved cancer detection equipment and technology.”

The ACDC at the H. Lee Moffitt Cancer Center and Research Institute has addressed these goals through studies that target the discovery of molecular and genetic markers of cancer risk, the identification of individuals at high risk for cancer through screening, and the testing of methods to prevent cancer. In addition, the ACDC created and supported education programs to provide increased cancer awareness, provide screening services, and has established working collaborations with the nearby James A. Haley VA Medical Center, the Bay Pines VA Medical Center and the MacDill Air Force Base Hospital.

The Advanced Cancer Detection Center supports research and demonstration projects that further its mission. Each project is reviewed for scientific merit by an internal peer group and an external scientific advisory committee. Preference is given to projects that have potential to lead to independent peer reviewed funding. During the current grant period, the ACDC supported eight cancer prevention and control research protocols. Four of these studies were continuations of projects begun in prior years and three are studies initiated the past year. The continuing studies are:

*Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers (Cohort Study),*

*The Specific Role of Isoflavones in Reducing Hormonal and Proliferative Risk Parameters in Prostate Cancer,*

*The Moffitt Cancer Network as a Telemedicine and Teleconferencing Educational Tool for Health Care Providers,*

*African American Families with Inherited Breast or Ovarian Cancer.*

*Molecular Fingerprint of STAT3 Regulated Genes for Early Detection of Human Cancer,*

*The Tampa Bay Ovarian Cancer Study (TBOCS),*

*Molecular Predictors of Disease Behavior in Thyroid Cancer, and*

*Bax-dependent Apoptosis in Prevention and Detection of Human Prostate and Lung Cancer.*



In November, 2000, both the internal and external scientific advisory committees met to consider the progress of the ACDC and to review and recommend new projects for funding. In this review, four additional projects were selected:

*Epoxide hydrolase genetic polymorphisms and their functional significance,  
Automated Quantified Screening for Melanoma,  
Breast Cancer Screening in High-Risk Women: Comparison of magnetic  
resonance imaging (MRI) with mammography, and  
Adaptive Computer Assisted Diagnosis (CAD) Method for Lung Nodule Early  
Detection.*

These studies were to be supported by FY00 and FY01 funding which was appropriated at the level of \$3.5 million for each year. On September 20, 2001 the U.S. Army Medical Research and Material Command, (MRMC) Ft. Detrick, MD awarded the ACDC \$6,004,000.00. According to the agency, this contract fully obligates the FY00 (\$3.5M) and FY 01 (\$3.5M) money directed to the ACDC by Congress. The remaining \$996,000.00 (14.2%) is being withheld by the agency for "overhead expenses." The delay in the obligation of these funds was attributed to a complex "RCQ" (Regulatory Compliance and Quality) approval process by the agency over "human use" issues such as human genetics. Accordingly, progress on these projects has been delayed. With the exception of the Epoxide hydrolase genetic polymorphisms and their functional significance project (PI: Dr. J. Park), which was approved as human subject exempt, the remaining projects are still undergoing RCQ review. Progress reports on these studies will be reported under the new award made from the ACDC. Indeed, only the Cohort and TBOC studies will receive continued funding from this ward in FY02. Final reports and publications are being readied on the remaining studies, as appropriate, or continuation funding is being developed under other mechanisms.

### **BODY:**

Overview: The H. Lee Cancer Moffitt Center & Research Institute includes a free standing patient care facility with a large inpatient and outpatient capacity, a major research institute consisting of more than 130 scientific members, a free standing Lifetime Cancer Screening Center and a wide array of outreach and educational activities for the general public and select underserved populations. Moffitt Cancer Center's location at the convergence of the University of South Florida's Health Sciences Center and the main campus sets the stage for the its conceptual commitment to interdisciplinary approaches to research and patient care. Moreover, it allows the Center to enjoy all intellectual advantages of a matrix center while remaining operationally freestanding. After 14 years, the Cancer Center's mission remains totally focused on "contributing to the prevention and cure of cancer."

The Cancer Center was created by the Florida Legislature in the early 1980s, to meet a clear and compelling need to respond to Florida's "cancer epidemic." Building a major cancer research and treatment center at the University of South Florida in Tampa was largely the vision of H. Lee Moffitt, a state legislator who served as Speaker of the

Florida House of Representatives from 1982-84. Construction of the original, 380,000 square foot hospital facility was funded with \$70 million from the state's cigarette tax, allowing the Center to open in 1986.

The initial phase of the Cancer Center's strategic plan called for a rapid and substantial deployment of its clinical, financial, and philanthropic resources to develop a true scientific center of excellence. The Center recruited Dr. John C. Ruckdeschel as the Cancer Center's first director in late 1991. In 1992, he began fulfilling that strategic plan, a process that culminated in the awarding of a Cancer Center Support Grant (CCSG) five years later.

The strategic plan's second phase continues the focus on scientific and clinical growth, with a commitment to increase research facilities by over 200,000 sq.ft., and to prepare to accommodate twice as many patients by 2009. In 1998, the state legislature committed an additional \$100 million to finance the construction needed to meet these goals.

#### Key Milestones 1997-2001:

- Increased peer reviewed funding from \$8 million in 1996 to over \$20 million in 2001.
- Increased NCI funding from \$4 million to \$12.1 million.
- Recruited more than 20 new basic and physician scientists
- Hosted or co-hosted national and international cancer conferences, with a major conference, *New Molecular Targets for Cancer Therapy*, conducted on October 14-18, 2000.
- Opened 45,000 sq. ft. of additional laboratory space, with the help of an NIH construction grant (RR 13592) to complete one floor.
- Successfully competed for two program project grants: "Cancer Drug Discovery: Cell Cycle Control Targets," P.I.: Said Sebti, Ph.D. (1 P01 CA78038-01); and "Molecular Oncology Program Project," PI: William Dalton, Ph.D., M.D. (1P01 CA82533).
- Increased the number of patients enrolled on clinical trials (all types) from 1,809 in 1996 to more than 3,700 in 2000.
- Became the 17<sup>th</sup> member of the National Comprehensive Cancer Network.
- Worked with the University of South Florida to develop an Interdisciplinary Oncology Program (Department of Oncology) that will include most of the Cancer Center's faculty and that allows a distinct practice plan arrangement with the USF College of Medicine. This Department includes the entire basic science faculty recruited by the Cancer Center and will be the academic home for a new interdisciplinary Ph.D. program in Molecular Oncology developed jointly by the Cancer Center and USF.

- Developed an Intellectual Property Sharing Agreement with the University of South Florida that gives the Cancer Center a percentage of all royalties and licensing fees on products developed by the Cancer Center's faculty.

- In September, 2000, the National Cancer Institute recommended renewal of the Cancer Center Support Grant for five years and the designation of Moffitt as an **NCI Comprehensive Cancer Center**, effective with its Notice of Award in February, 2001.

Today, the Cancer Center's membership numbers 130 scientists and clinicians who are USF faculty. More than 94 members-in-residence are housed and supported in the Center's facilities and work under the terms of the USF/Moffitt affiliation and faculty support agreements. Other members are based in University departments. The Cancer Center's 1,500 employees support the work of the physicians and scientists. The Center has annual operating revenues of over \$130 million yearly, including an \$11 million annual appropriation from the State of Florida, research grants totaling more than \$26 million overall (direct), philanthropic donations, and institutional commitment from the University of South Florida in the form of faculty salaries and a portion of clinical practice revenues.

The Cancer Center currently supports four scientific programs:

<u>Program</u>	<u>Leader</u>	<u>Members</u>	<u>Funding (Direct)</u>
Molecular Oncology	Richard Jove, Ph.D.	21	\$ 4,466,000
Immunology	Julie Djeu, Ph.D.	14	\$ 2,486,000
Clinical Investigations	William Dalton, Ph.D., M.D.	58	\$ 7,929,000
Cancer Control	Jeffrey Krischer, Ph.D.	39	\$ 8,163,000
Non-aligned members & institutional grants	N/A	5	\$ 3,089,000

The Cancer Control Research Program is the largest research program at Moffitt with 38 active members and more than \$8 million in research funding. The overall goals of the Cancer Control Research Program remain focused on the reduction of the burden of cancer on individuals and society. The goals of the Cancer Control Research Program are translated into specific focused scientific aims that can be summarized as the application of multidisciplinary research to:

- |                       |  |
|-----------------------|--|
| Aim 1 Susceptibility  | Identify markers that predict increased cancer susceptibility.                                 |
| Aim 2 Prevention      | Evaluate promising interventions directed at the prevention of cancer.                         |
| Aim 3 Early Detection | Develop and testing new early detection strategies.  |
| Aim 4 Health Outcomes | Evaluate interventions to improve the quality of life for cancer patients & their care-givers. |

These aims are consistent with those of the Advanced Cancer Detection Center and the funding has been utilized to create an infrastructure to promote the goals of the Cancer Control Research Program by:

- Encouraging collaborative research
- Providing funding for studies that can lead to extramural peer-reviewed funding
- Providing core competencies to support Cancer Control investigators

In order to provide an appropriate mechanism to allocate and manage these funds, the Cancer Center created an administrative core, an internal scientific review committee, and an external advisory committee. The administrative core manages the resources and personnel, associated with the ACDC funding, and provides liaison with the Department of the Army and the regulatory bodies that oversee the research. The internal scientific review committee conducts a scientific review of the merits of proposed projects and their potential for peer-reviewed funding and makes funding recommendations. The external advisory committee reviews the organizational structure and scientific directions of the Advanced Cancer Detection Center and the progress made by the individual projects.

The membership of the internal scientific review committee changes as necessary to have adequate scientific expertise to evaluate proposals submitted to the ACDC. This year the members are:

Dr. Dmitry Goldgof, Associate Professor, Computer Science and Engineering,  
College of Engineering

Dr. Pamela Munster, Assistant Professor, Department of Interdisciplinary Oncology,  
College of Medicine

Dr. Santo Nicosia, Professor and Chair, Department of Pathology, College of  
Medicine

Dr. Robert Clark, Professor and Chair, Department of Radiology, College of  
Medicine

Dr. Jeffrey Krischer, ex officio, Professor, Department of Interdisciplinary Oncology,  
College of Medicine

Cancer Control science at the H. Lee Moffitt Cancer Center and Research Institute is greatly enhanced and facilitated by the development of infrastructure that provides access to shared resources, promotes collaboration and funds pilot projects. Over the last three years, the Cancer Control Program has established new infrastructure to meet these needs. The funding of the Advanced Cancer Detection Center is one of three mechanisms by which this has occurred.

#### **Advanced Cancer Detection Center**

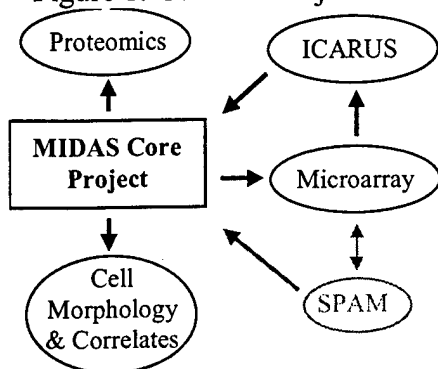
The Advanced Cancer Detection Center has become a significant component of the Moffitt Cancer Control Program infrastructure in that provides a stimulus for research development and promotes inter and intra programmatic collaborations. The Advanced

Cancer Detection Center supports pilot studies that can lead to peer-reviewed extramural funding. Projects supported by this mechanism follow a two-tiered scientific review process in which the science and the likelihood of peer-reviewed extramural funding are considered. In addition, priority is given to projects that foster inter and intra-programmatic collaborations.

### **Center for Mathematical-Modeling of Image Data Across the Sciences (MIDAS)**

**(PI:Lazaridis)**, through a two-year competitive grant jointly funded by the Moffitt Cancer Center and the University provost's office in June, 1999. The mission of the MIDAS center is to create interdisciplinary collaborations among imaging, quantitative and biological scientists, in order to develop new analytic models of image-related data and to train researchers of various disciplines in modeling techniques.

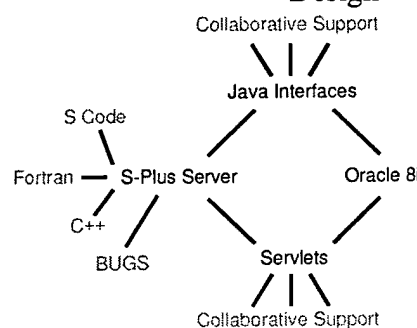
Figure 1: MIDAS Projects



The Center is comprised of a core project and several satellite projects. The core project is to construct a data analytic environment that builds advanced statistical methodologies on top of available imaging componentry. By borrowing and uniting technologies from multiple fields, we are seeking to empower researchers in both basic and clinical imaging studies with a sophisticated analytic toolbox. Because this core project is a prerequisite technology for a center focused on

quantitative, collaborative analyses of image-related data, substantial resources have been committed to its completion, including hardware, software, and the efforts of two statistical assistants and data systems programmers. Multiple Java interfaces and servlets are being built from reusable object-oriented code to tie together powerful statistical and database systems (Figure 2). Graduate students and postdoctoral fellows participating in the satellite projects also contribute to the core project. New methods have been developed and a patent application is pending. The applied projects entail interactions with investigators in Molecular Oncology, Clinical Investigations as well as Cancer Control.

Figure 2: MIDAS Core Project Design



### **Moffitt CCOP Research Base (PI:Krischer)**

The H. Lee Moffitt Cancer Center received funding by the NCI in June 2000 to develop a research base as a mechanism for Community Clinical Oncology Programs to access cancer control clinical trials. NCI CCOPs and Moffitt affiliates are eligible to participate in the Moffitt CCOP Research Base. Membership is based on continued funding as an NCI CCOP with satisfactory performance measured by accrual and data quality.

The goals of the Moffitt CCOP Research Base are to:

- Develop cancer control trials of high scientific merit for implementation in the community setting.
- Provide community investigators an opportunity to participate in NCI-supported cancer control clinical trials.

The following CCOPs have, or are in the process of, establishing formal affiliations with the Moffitt CCOP research base:

Florida Pediatric CCOP, Tampa, FL  
 Merit Care Hospital CCOP, Fargo, ND  
 Mount Sinai Medical Center CCOP, Miami, FL  
 South Texas Pediatric MBCCOP, San Antonio, TX  
 Baptist Center Research Institute CCOP, Memphis, TN  
 Cancer Research for the Ozarks CCOP, Springfield, MO  
 Columbus CCOP, Columbus, OH  
 Greater Phoenix CCOP, Phoenix, AZ  
 North Shore University Hospital CCOP, Manhasset, NY  
 NorthWest CCOP, Boise, ID  
 Southern Nevada Cancer Research Foundation CCOP, Las Vegas, NV

The Moffitt CCOP Research Base is now staffed and cancer control protocols and concepts are being initiated. Several of the clinical studies are the result of pilot development funded by ACDC projects:

A Clinical Trial of the Action of Isoflavones in Breast Neoplasia: Administration Prior to Mastectomy or Lumpectomy -- A Pilot Study	Protocol	Pending 10/17/2001
The Specific Role of Isoflavones in Reducing Prostate Cancer Risk	Protocol	Activated 09/05/2001
A Randomized Pilot Clinical Trial of the Action of Isoflavones and Lycopene in Localized Prostate Cancer: Administration Prior to Radical Prostatectomy.	Protocol	Pending 09/06/2001
Megestrol Acetate (Megace) as an Appetite Stimulant in Children	Protocol	Activated 02/27/2001
Cancer Genetic Counseling and Testing by Telemedicine in Community Settings	Concept	Pending
Methylphenidate for the Treatment of Cognitive Difficulties in Patients with Primary Brain Tumors: A Double-Blinded, Placebo-Controlled, Parallel Group Study	Concept	Pending

### **(1) KEY RESEARCH ACCOMPLISHMENTS:**

The material that follows in this section summarizes the key research accomplishments associated with each project and task outlined in the appropriate approved Statement of Work for ACDC approved projects. **A full description of the projects and their progress is appended.**

#### **Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers, Genetic Analysis of Familial Prostate Cancer**

- Developed an infrastructure to identify, accrue, screen and follow a non-diseased community-dwelling population at high risk for lung cancer.
- Developed procedures for collection and preservation of sputum specimens for new (DNA, RNA, protein and morphologic) markers of pre-neoplasia.
- Developed an archive of airways cytologic specimens suitable for evaluation of new (DNA, RNA, protein and morphologic) markers of pre-neoplasia.
- Developed an archive of white blood cells suitable to provide individual control specimens for DNA and RNA
- Developed a potency assay for MoAb 703D4 immunodetection of hnRNP A2/B1 protein expression.
- Identified a panel of 11 LOH markers for sputum lung cancer screening which identifies 84% of lung tumors (See appendix table III).

#### **The Specific Role of Isoflavones in Reducing Hormonal and Proliferative Risk Parameters in Prostate Cancer**

As planned and outlined in our Statement of Work, we have accomplished the following:

##### **Task 1: Recruitment and Data Collection:**

- a. We have pre-screened 120 subjects and recruited 69 eligible patients diagnosed with grade 1-2 prostate cancer who were consecutively admitted to the Prostate Program. This was the recruitment completed for the pilot phase of the trial. Thirty one (31) subjects have completed the study and an additional 20 are active in this study. Eighteen subjects dropped out of the study as they were not able to consume the soy and placebo supplementation.
- b. Upon eligibility, consent was obtained from all subjects. Upon enrollment, the following baseline information has been obtained from all subjects admitted to the study.(Study Schema- Figure 1)

Study Schema – Table-1

Study Requirements	Baseline	During Intervention	Post-Intervention (end of 12 weeks or Off Study)
Anthropometric measurements (Weight, Height)	X	Every 4 weeks	X
Two Day Food Record (TDFR) <sup>1</sup>	X	Weekly	X
Sign informed consent	X		
Eligibility form	X		
History: (On Study form) Demographic information Personal & medical history Hormonal & reproductive history Exercise Smoking Alcohol use	X		
Non-fasting blood samples:			
Hormonal assays <sup>2</sup>	X		X
Total prostate specific antigen <sup>2</sup>	X		X
Study agent intake log		Daily	X
Clinic visit: Obtain study agent/return left over study agent Review completed TDFR and daily intake log		Every 4 weeks	X

1. Confirmation of the accuracy of eligibility information, including the 4-day diet records and using an initial screening form (Baseline only)
2. Demographic information, personal and medical history, hormonal and reproductive history, exercise, smoking and alcohol use history obtained by an RD using the Epidemiological Questionnaire (Baseline only).
3. Anthropometric measurements such as subject's height, weight, skinfold and circumference measurements (Baseline, week 6 and 12)
4. 30 mL Blood samples will be drawn into heparinized tubes in a non-fasting state at the same time of day, between 7:00 PM and 12:00 AM, for each individual to perform hormonal assays and total and percentage free prostate specific antigens.(Baseline, week 6 and 12). Hormonal assays will include free testosterone, sex-hormone binding globulin and estradiol at baseline, week 6 and at week 12. As there are no previous studies that have established the duration required to demonstrate change in hormonal levels with intake of isoflavones in males, we adopted the time taken to demonstrate hormonal changes with ingestion of isoflavones in female populations which is within one menstrual cycle. We had thus established the evaluation point as 12 weeks or 3 months for both the female and male groups. The hormonal assays (radioimmunoassay) will be performed by Quest Laboratories. Blood draws are done by the Cancer Center phlebotomist and



- processed and shipped using standard procedures for shipping to Corning Nichols, who will perform the radioimmunoassays.
5. A biopsy and digital rectal exam will be performed by the GU program chief/oncologist for all patients entered in the study at baseline(routine),which will determines patient's admissibility to the study.
  6. The participant will be provided with a 2-day diet record(TDFR) and instructed on reporting food intake, including weights/measures and methods of preparation of foods consumed using standard food models. (Baseline, weekly)
  7. Changes may be anticipated in stool frequency or GI discomfort. A pre-validated Nutritional Symptoms Scale is used to monitor GI symptoms during intake of supplements on a weekly basis.
  8. A Participant Tracking Form is used to monitor all activities and variables observed during the study period. Activities of each participant is vital for the study such as use of supplements, compliance to all monitors. This form, will in addition, serve as a checklist to monitor these variables for the Project Dietitians.
  9. Quality control procedures for data collection and entry are ongoing.
  10. Contact numbers were provided to patients

Task 2: Abstraction of Medical Records Data:

- a. We have continued to obtain patient disease related prognostic indicators from medical charts
- b. Data entry and quality control procedures have been initiated
- c. Follow-up interviews for data collection periods at mid-point and post completion of interventions have been completed for 31 subjects and the currently active 20 subjects is in progress.
- d. Weekly visits to the cancer center to obtain supplements and submission of monitoring instruments
- e. Shipping of completed patient's blood sample for hormonal assays and PSAs are ongoing

At the end of the study we will complete the data analysis. Pooled t-tests will, in addition be used to compare mean changes in intake of other nutrients, body composition parameters and nutritional symptoms at the end of Phase II. Pooled t-tests are justified in this case. Even if the data are only approximately normally distributed, the test is well known to be quite robust with respect to the normality assumption. These tests will be two-sided.

Multivariate repeated measures ANOVA will be performed on each variable with time as the repetition variable and treatment group as class variable. "Time effect", "group effect" and "time by group" interaction will be tested. If this last effect is significant, then "time effect" will only be reported separately for each group and "group effect" will only be reported separately for each time. The need to adjust for multiple testing is somewhat controversial. We will exercise some control over the multiple testing problem by comparing groups at each time point only if the overall ANOVA is significant. We recognize that this analysis may have limited power due to missing data. For that reason

the repeated measures ANOVA will be considered a secondary analysis and the methods described previously will be primary.

Preliminary results of the data collected from the pilot phase of the study are as follows:

- We have recruited 69 eligible subjects for the isoflavones/prostate cancer study and all have completed the study. The randomized clinical trial has been initiated and 31 subjects have completed the study and 20 are currently active in the study. A total of eight (18) subjects dropped out of the study, 16 of whom were unable to tolerate the taste of the product, and 2 dropped out as they experienced constipation. Due to the large number of drop-outs in the study, we are currently oversampling and recruiting additional subjects in this study.
- The average age of the isoflavone group was 73.7 and the placebo group 70.8. No significant differences in baseline height, weight, Body Mass Index (BMI), smoking, family history of cancer was observed.
- No significant difference between the groups was observed on the basis of the Baseline Nutritional Intake of calories, fats, proteins, carbohydrates, fiber and cholesterol of both the experimental and placebo groups is displayed in Table 2.
- Total PSA decreased an average of over 2 points in 69% of subjects in the isoflavone group compared to no decreases seen in subjects in the placebo group.
- Free testosterone decreased in 56% of subjects in the experimental group compared to 27% of subjects in the placebo group. We observed an increase in total estradiol levels in the group receiving isoflavones compared to a decrease in this marker observed in the placebo group. However, no significant changes were observed in SHBG levels.

#### **Development of the Moffitt Cancer Network**

- The Moffitt Cancer Network is available to users and can be found at <http://network.moffitt.usf.edu>
- The MCN currently has 270 presentations in its library, increasing at a rate of 8.8 presentations per month on average. Additionally, 11 conferences sponsored by USF and Moffitt are also currently available online.
- Increase in average accrual rate from 2.3 presentations per month to 8.8 presentations per month over the past year.
- All approved Grand Rounds presentations have been taped by the Moffitt Multimedia Education Resources Center (MERC) for over two years preceding this report. The video had been captured on digital DVCAM 94 minute tapes. Recently, we have moved to a tapeless acquisition process.
- Since many of the presenters use only 35mm slide for their presentations, a process of creating final production audio/video Real media for streaming via TCP/IP has been developed. This process requires post-production labor and requires the best of the video's individual frames to be captured a second time to recreate higher quality computer images. MCN has made significant progress in this area and as of June

2000 has begun using presenter's PowerPoint files when ever possible to bypass the second image rendering process. This has reduced labor time from 3.5 days to about 5 hours, while increasing image quality noticeably. This labor savings is not realized when presenters are using 35mm film only. MCN is exploring new avenues to further automate the process while increasing quality.

- National oncology conferences have been taped and included in the MCN website database.
- Conferences have been subdivided into their respective presentations and are categorized searchable as well as searchable using the website database Access Jet engine. All conferences are pre-qualified for their ability to become online educational materials by the University of South Florida College of Medicine and, more recently, the University of South Florida College of Nursing. As of October 2000, we are able to issue credit for conference presentations.
- MCN is successfully using Microsoft MPEG-4 streaming. Not yet standardized, the newly introduced streaming format allows for embedded script and control processes within the media stream while decreasing bandwidth requirements and increasing quality.
- MCN has begun a process of evaluating user behavior patterns in efforts to work towards an "intelligent website".

#### **African American Families with Inherited Breast or Ovarian Cancer.**

- Aim 1: Mutations were identified in 2 of 8 families tested.
- Aim 2: BRCA2-associated tumor was ER/PR+; BRCA1-associated tumor was ER/PR-  
The two families with mutations had the strongest family history/highest risk estimates.
- Aim 3: Baseline awareness of inherited cancer susceptibility was limited.

#### **Molecular Fingerprint of STAT3 Regulated Genes for Early Detection of Human Cancer**

- Identified 303 unique mouse genes which display altered regulation by v-Src in the context of STAT3 activation and oncogenesis.
- Hierarchical clustering illustrates that gene expression patterns of v-Src transformed cell lines with activated STAT3 cluster together regardless of cell type.
- Identified 631 human genes that display altered expression associated with STAT3 activation in breast cancer.
- Implemented and tested software that extracts raw data from Affymetrix files.
- Developed and published a novel approach for obtaining gene expression estimates from raw probe set data.
- Constructed an enterprise-capable interface and data management system for Affymetrix data.

### **The Tampa Bay Ovarian Cancer Study**

- Aim 1: We are successfully collecting data regarding health behaviors and risk factors from all participants via questionnaire instruments and study interview.
- Aim 2: We are successfully collecting detailed cancer family history from all participants via questionnaire instruments and study interview.
- Aim 3: We have established a successful mechanism to obtain medical records and tumor tissue in order to compare tumor characteristics between mutation-associated cases and non-mutation controls.
- Aim 4: We have established a successful follow-up mechanism to obtain data regarding differences in response to treatment and survival between mutation-associated cases and non-mutation controls.
- Aim 5: We are implementing additional strategies to achieve 80% participation of eligible cases and anticipate success by summer 2002.

### **Molecular Predictors of Disease Behavior in Thyroid Cancer**

Nothing to report until full analysis is done.

### **Significance of Bax-dependent Apoptosis in Prevention and Detection of Human Prostate and Lung Cancer**

- Bax is required for a peptidyl proteasome inhibitor-induced apoptosis in human prostate or lung cancer cells.
- Bax is required for tea polyphenol EGCG-induced apoptosis in human prostate or lung cancer cells.
- Resistance of human normal lung cells to proteasome inhibitor treatment is associated with failure of these cells to accumulate Bax to mitochondria.
- Decreased Bcl-2 expression in mitochondria sensitizes tumor cells to induction of apoptosis.

### **(2) REPORTABLE OUTCOMES:**

Manuscripts, abstracts, presentations:

### **Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers**

Publications Related to this Study:

1. Chirikos T, Hazelton T, Tockman MS, and Clark R. Screening for Lung Cancer with CT: A Preliminary Cost-Effective Analysis. CHEST. In Press.
2. Zhou J, Nong L, Wloch M, Cantor A, Mulshine JL, Tockman MS. Expression of early lung cancer detection marker: hnRNP A2/B1 and its relation to microsatellite alteration in non-small cell lung cancer. Lung Cancer. 2001. In press.

3. Mulshine JL, De Luca LM, Dedrick RL, Tockman MS, Webster R, Placke ME. Considerations in developing successful, population-based molecular screening lung cancer. *Cancer*. Dec 1, 2000; 89(11 Suppl):2465-7. Review.
4. Tockman MS, Mulshine JL. The early detection of occult lung cancer. *Chest Surg Clin N Am*. Nov 2000; 10(4): 737-49.
5. Tockman MS. Advances in Sputum Analysis for Screening and Early Detection of Lung Cancer. *Cancer Control, Journal of the Moffitt Cancer Center*. January/February 2000, Vol. 7, No. 1:19-24.
6. Kennedy TC, Proudfoot SP, Piantadosi S, Wu L, Saccomanno G, Petty TL, Tockman MS. Efficacy of Two Sputum Collection Techniques in Patients with Air Flow Obstruction. *ACTA Cytologica*. July-August 1999; Vol. 43, No. 4:630-636.

#### Abstracts Related to this Study:

1. Zhukov TA, Erozan YS, Tockman MS. Sentinel Cell for Lung Cancer. 14<sup>th</sup> International Congress of Cytology, RAI Congress Centre, Amsterdam, The Netherlands. May 2001.
2. Mulshine JL, Tockman MS, Martinez A, Man Y-G, Montuennnga L, Hong SH. Application of Molecular Biology for Early Detection of Lung Cancer. 1999.
3. Zhou J, Nong L, Wloch M, Zhukov TA, and Tockman MS. Expression of Early Lung Cancer Detection Marker: hnRNP a2/b1 and its Relation to Microsatellite Instability in Non-Small Cell Lung Cancer. *Proc. AACR*, April 1999, 40:140-141
4. Truncate T, Zhou J, Zhukov TA, Muñoz-Antonia T, Antonia S, Muro-Cacho C, Tockman MS. Evaluation of TGF- $\beta$  II Receptor Expression (TBR-II) and a Common Signaling Mediator SMAD4 in NSCLC. *Proc. AACR*, April 1999, 40:337.
5. Falestiny MN, Cardona JJ, Zhou J, Zhukov TA, Nong L, Solomon DA, Tockman MS. Transforming Growth Factor  $\beta$  Type II Receptor Expression in Non-Small Cell Lung Cancer, Viral Transformed Bronchial Cells and Normal Bronchial Epithelial Cells: A Comparative Study. *Chest (Suppl)*; November 1998.
6. Tockman MS, Saccomanno G, Michels R, Zhukov TA, Erozan Y, and Gupta P. Sentinel Cell for Lung Cancer. 6<sup>th</sup> SPORE Investigator's Workshop; July 1998.

#### Presentations Related to this Study:

- |                     |   |
|---------------------|---|
| December 8-10, 1998 | International Conference on Prevention and Early Diagnosis of Lung Cancer, Johns Hopkins Lung Project and Immunocytochemical Screening for Lung Cancer. University of Varese and University of Massachusetts Medical School, Varese, Italy. |
| February 12, 1999   | ALCASE Workshop – Lung Cancer: A Revolution in Care, Technology in Early Diagnosis of Lung Cancer. Embassy Suites, Tampa, Florida   |
| April 26, 1999      | 1999 ALA/ATS International Conference Program, Early Sputum Marker for Lung Cancer (hnRNP). San Diego Convention Center, San Diego, California  |

April 30, 1999	Pharmacology Seminar Program, Detection and Immunostaining of the Lung Cancer Sentinel Cell. University of Pittsburgh, Pennsylvania.
September 13, 1999	Advanced Cancer Detection Center, External Advisory Committee. Moffitt Cancer Center, Tampa, Florida
September 30 to October 3, 1999	The First International Conference On Screening for Lung Cancer, Cornell University, New York
October 9-13, 1999	Annual Congress of the European Respiratory Society, Dysregulation of the Cell Cycle in Lung Cancer. Madrid, Spain
October 15-16, 1999	Molecular Biomarkers Workshop, Roy Castle Lung Cancer Foundation, Liverpool, England
October 26, 1999	Screening of Lung Cancer Conference, Gaithersburg, MD
October 31, 1999	7 <sup>th</sup> Annual Scientific Assembly of the American Association of Bronchology, New horizons in cytological based early detection in lung cancer. Chicago, IL
February 9, 2000	International Symposium on Early Detection of Lung Cancer, Molecular Screening Program: Past, Present, and Future. Tel Aviv, Israel
February 27-29, 2000	International Agency for Research on Cancer, Use of Biomarkers in Chemoprevention of Cancer, Lung Cancer: Intermediate Effect Markers. Heidelberg, Germany
March 20, 2000	Cahan Lectureship at Memorial-Sloan Kettering, Molecular Screening for Lung Cancer. New York, NY
April 12, 2000	Early Detection Research Network Site Visit at H. Lee Moffitt Cancer Center & Research Institute, Organization of BeDLAM. Tampa, FL
June 16, 2000	Wayne State University Cancer Conference, Sputum in 2000: Hypothetical Advantages, Practical Limitations, and Novel Approaches, Detroit, MI
June 22, 2000	Reducing Lung Cancer Mortality: Actions for the New Millenium, Sputum Based Detection of Preinvasive Lung Cancer, Washington, DC
June 27, 2000	Roy Castle Lung Cancer Foundation and H. Lee Moffitt Cancer Center, Quest for the Cure, Lung Cancer Screening and Early Detection: Spiral CT Scanning and Molecular Markers, Tampa, FL
July 19, 2000	H. Lee Moffitt Cancer Center/USF Lung Cancer Conference, Epidemiology and Early Detection of Lung Cancer, Coeur d'Alene, ID
July 19, 2000	H. Lee Moffitt Cancer Center/USF Lung Cancer Conference, The Management of Pre-Clinical Lung Cancer, Coeur d'Alene, ID
September 12, 2000	IASLC 9 <sup>th</sup> World Conference on Lung Cancer, Cellular Targeting in the Molecular Diagnosis of Lung Cancer, Tokyo, Japan

October 24, 2000	66 <sup>th</sup> International Scientific Assembly of the ACCP, San Francisco, CA ACCP Post Graduate Course, Screening and Early Detection of Lung Cancer Meet the Professor, Sputum Detection of Early Lung Cancer: Hypothetical Advantages, Practical Limitations, and Novel Approaches
October 27, 2000	Cornell CT Conference, Sputum Detection of Early Lung Cancer: A Compliment to Helical CT, New York, NY
March 7-8, 2001	Lung Cancer Early Detection Workshop, National Cancer Institute/ American Cancer Society, "New Frontiers of Screening Science". Rockville, MD
March 24-25, 2001	Second Annual – A Practical Pulmonary Review for Primary Care Providers, University of South Florida/Department of Veterans Affairs, James Haley, "Early Recognition of Lung Cancer". St. Pete Beach, FL
June 20-22, 2001	Early Detection Research Network, National Institute of Health/National Cancer Institute, "Lung Cancer Screening Update" and "Industrial Partnership with EDRN", Washington, DC
June 26-July 2, 2001	Second International Lung Cancer Molecular Biomarkers Workshop "A European Strategy for Developing Lung Cancer Molecular and Clinical Diagnostics in High Risk Populations, Roy Castle Lung Cancer Foundation, "Sputum in 2001: Hypothetical Advantage Practical Limitations, and Novel Approaches" and "Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers" Liverpool, England
August 7-12, 2001	3 <sup>rd</sup> International Conference on Prevention & Early Detection of Lung Cancer, International Association for the Study of Lung Cancer, "Cellular Approaches to Lung Cancer Detection" and "Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers". Reykjavik, Iceland

**The Specific Role of Isoflavones in Reducing Hormonal and Proliferative Risk Parameters in Prostate Cancer**

Abstracts and Presentations Related to this Study:

1. The Specific Role of Genistein in reducing hormonal and proliferative risk parameters in Prostate Cancer. Kumar NB, Pow-Sang J, Besterman-Dahan, K, Cantor A, Seigne J & Allen K. Proc 10<sup>th</sup> Annual Research Conference, American Institute for Cancer Research, August 2000.
2. The Specific Role of Genistein in reducing hormonal and proliferative risk parameters in Prostate Cancer. Kumar NB, Pow-Sang J, Besterman-Dahan, K, Cantor

A, Seigne J & Allen K. Proc. of the 4th Annual Symposium on Predictive Oncology and Therapy sponsored by the International Society for Preventive Oncology; 2000.

**African American Families with Inherited Breast or Ovarian Cancer**

Pilot data from this study will be used in a funding application for a more extended project through the Florida Cancer Genetics Network.

A manuscript is in preparation.

**Molecular Fingerprint of STAT3 Regulated Genes for Early Detection of Human Cancer**

An abstract was submitted to the Oncogenomics meeting held in Tucson, Arizona, in January of 2001. This meeting was sponsored by the American Association for Cancer Research and *Nature Genetics*, and is one of the major international meetings on the application of microarray gene expression profiling to cancer. Our abstract was one of the few selected for a platform talk. An abstract was also submitted to the Oncogene meeting held in Fredrick, Maryland in June 2001, and selected for a poster presentation. Both abstracts, which are attached as appendixes, were presented by Dr. Dominic Sinibaldi, who is the Postdoctoral Fellow bridging the research groups of Dr. Jove and Dr. Lazaridis on this project by performing laboratory experiments and participating in the data analysis.

The manuscript, "A Simple Method to Improve Probe Set Estimates from Oligonucleotide Arrays", has been accepted for publication in *Mathematical Biosciences*.

An NIH R01 grant application is currently in preparation to extend the studies funded by this ACDC/DOD project. The application will have Dr. Jove as the Principal Investigator and Dr. Lazaridis as a Co-Investigator and will be submitted for November 1, 2001. We anticipate that the data analysis to define the final STAT3 molecular signature will be completed within 3 months and that a manuscript describing this work will be submitted for publication in January, 2002. Dr. Dominic Sinibaldi has received outstanding training in cutting-edge molecular biology and bioinformatics while working as a Postdoctoral Fellow on this project, which has prepared him well for assuming a leadership position in the field as an independent investigator.

**The Tampa Bay Ovarian Cancer Study**

Based on the epidemiologic design of the Tampa Bay Ovarian Cancer Study, funding was awarded by the American Cancer Society for a 3-year companion study to evaluate the role of biologically active lysophospholipids for their potential as biomarkers of ovarian cancer. Preliminary data is promising and shows that certain lysolipids appear to be elevated in the plasma of women with ovarian cancer compared with healthy controls. Based on this preliminary data, we have applied for an R01 to investigate the use of lysolipid measurement for detection of ovarian cancer in a population of women at increased risk of ovarian cancer, including first-degree relatives of women in TBOCS (ovarian cancer patients). Our ongoing contact with women in TBOCS will facilitate the identification and enrollment of their female relatives at increased risk for enrollment in this important study, toward the development of an early detection test for ovarian cancer.



Based on data showing that gene mutations associated with Hereditary Non-Polyposis Colorectal Cancer (HNPCC) are the third leading cause of hereditary ovarian cancer (after BRCA1 and BRCA2), and the suggestion that ovarian cancer is a “sentinel cancer” in individuals with these gene mutations, funding for the investigation of HNPCC as a companion study of TBOCS has been proposed.

#### **Molecular Predictors of Disease Behavior in Thyroid Cancer**

None at this time.

#### **Significance of Bax-dependent Apoptosis in Prevention and Detection of Human Prostate and Lung Cancer**

1. Nam S, Smith DM and Dou QP. Inhibition of proteasome activity in vitro and in vivo by ester bond-containing tea polyphenols. J. Biol. Chem., 2001; 276: 13322-13330
3. Gao G and Dou QP. G1 phase-dependent Bcl-2 expression correlates with chemoresistance of human cancer cells. Mol. Pharm., 2000; 58: 1001-1010
3. Dou QP and Nam S. Proteasome inhibitors and Their Therapeutic Potential (invited review). Expert Opinion on Therapeutic Patents, 2000; 10: 1263-1272
4. Nam S, Smith DM and Dou QP. Dietary tannic acid potently inhibits tumor cell proteasome activity, increases p27 and Bax expression, and induces G<sub>1</sub> arrest and apoptosis. Cancer Epidemiology, Biomarkers & Prevention, in press

Three Abstracts are presented in the following meetings:

Smith DM and Dou QP. Green tea targets human tumor cell DNA synthesis and consequently induces apoptosis. Poster presentation. New Molecular Targets for Cancer Therapy, St. Petersburg Beach, FL, October 14-17, 2000

Smith DM, Nam S and Dou QP. Studies on tumor related targets of green tea polyphenols. Poster presentation. 2<sup>nd</sup> International Conference. Mechanisms of Cell Death and Disease: Advances in Therapeutic Intervention, North Falmouth, MA, June 2-6, 2001

Kazi A, Smith DM, Zhong Q and Dou QP. Down Regulation of Bcl-X<sub>L</sub> Protein Expression by Green Tea Polyphenols Is Associated with Prostate Cancer Cell Apoptosis. Poster presentation. AACR-NCI-EORTC. Molecular Targets and Cancer therapeutics, Miami Beach, FL, October 29-November 2, 2001

- One Ph.D. degree will be obtained (David M. Smith is expected to graduate in Spring 2002), which is partially supported by this Award.

#### **Patents and licenses applied for and/or issued:**

#### **Development of the Moffitt Cancer Network**

- A patent covering the software developed for the Moffitt Cancer Network, that was developed under the project “The Moffitt Cancer Network as a Telemedicine and Teleconferencing Educational Tool for Health Care Providers,” is being pursued. A

notice of disclosure has been filed with the University of South Florida office of patents in anticipation of the completion of a patent application.

- **Funding applied for based on work supported by this award:**

**Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers**

- "The Biomarker Development Laboratory at Moffitt" (NCI-CA 84973, M. Tockman, PI, 1<sup>st</sup> year/Total award \$413,720/\$1,903,827).
- "Identification of the lung cancer epitope identified by the monoclonal antibody 703D4" (Cancer Research Foundation of America, M. Gruidl, PI, Total award \$38,950).
- NCI-EDRN, J. Park, PI; Total award \$98,000.

**The Tampa Bay Ovarian Cancer Study**

- Based on the epidemiologic design of the Tampa Bay Ovarian Cancer Study, funding was awarded by the American Cancer Society for a 3-year companion study to evaluate the role of biologically active lysophospholipids for their potential as biomarkers of ovarian cancer. Preliminary data is promising and shows that certain lysolipids appear to be elevated in the plasma of women with ovarian cancer compared with healthy controls. Based on this preliminary data, we have applied for an R01 to investigate the use of lysolipid measurement for detection of ovarian cancer in a population of women at increased risk of ovarian cancer, including first-degree relatives of women in TBOCS (ovarian cancer patients). Our ongoing contact with women in TBOCS will facilitate the identification and enrollment of their female relatives at increased risk for enrollment in this important study, toward the development of an early detection test for ovarian cancer.

**Significance of Bax-dependent Apoptosis in Prevention and Detection of Human Prostate and Lung Cancer**

- American Institute for Cancer Research. Tea Polyphenols Target Proteasome-Mediated Bax Degradation Pathway: Significance in Prostate Cancer Prevention and Treatment. Principal Investigator: Q. Ping Dou (15%). 02/01/01-01/31/03. Total Direct Costs: \$150,000; (invited for a resubmission).
- NIH R01. Synthetic EGCG Analogs and Prostate Cancer Prevention. Principal Investigator: Q. Ping Dou (25%). 07/01/02-06/30/07. Total Direct Costs: \$1,250,000;
- Tea Targeting Proteasome: A Role in Cancer Prevention (Determine potency, selectivity and effects of EGCG and EGC to inhibit the proteasome, the Bax degradation, activity in prostate cancer cell extracts and cell cultures; Principal Investigator: Q. Ping Dou, Ph.D. Agency: NIH/NCI; Type: R03 (CA091282-01, Years 1-2); Period: April 1, 2001 to March 31, 2003.

(3) **CONCLUSIONS:** The Advanced Cancer Detection Center has been a great success. It has attracted quality research projects from among Cancer Center members, it has promoted inter and intra programmatic research and its projects have begun to lead to peer-reviewed extramural funding. Two of these studies (Selenium, DAMD 17-00-1-0062, and the Moffitt Cancer Network, DAMD 17-00-1-0055) received independent peer-reviewed funding from the Department of Defense in February, 2000. Also, the study of the specific role of genistein in reducing hormonal and proliferative risk parameters in prostate cancer received supplemental peer-reviewed funding from the American Institute for Cancer Research, (PI:Kumar) and a randomized phase III clinical trial of the antioxidants isoflavones & lycopene in prostatic neoplasia (localized prostate cancer and high grade PIN) has been approved for funding under the auspices of the National Cancer Institute-funded Moffitt CCOP Research Base, (PI: Krischer). Based on the epidemiologic design of the Tampa Bay Ovarian Cancer Study supported by this award, funding was awarded by the American Cancer Society for a 3-year companion study to evaluate the role of biologically active lysophospholipids for their potential as biomarkers of ovarian cancer. The study of markers of transformation in airways epithelial cells from a cohort of obstructed smokers and former smokers supported the establishment of the Biomarker Development Laboratory at Moffitt also funded by the National Cancer Institute, (PI: M. Tockman,). The Cohort Study is the only study in the nation that currently evaluates both molecular airways markers and helical CT examinations simultaneously in the prospective detection of lung cancer. Until the Cornell and Mayo studies begin their collection of sputum specimens, no other study addresses the relative merits of these apparently complementary techniques for lung cancer screening. This research question addresses the most common cause of cancer death and the only common cancer for which no screening is available. Finally, the archive of radiographs, sputum and blood cell specimens provides an infrastructure for other investigators at Moffitt and across the nation. The recent award to Dr. Jong Park of an NCI Early Detection Research Network grant was based on the availability of the Cohort archive.

The current funding period has been extended to permit the successful conclusion of projects that are still in the accrual or analysis phases. Manuscripts, presentations and grant proposals are under development to communicate the results of these efforts widely and to secure additional funding to pursue promising findings. The Moffitt Cancer Network is a functioning, stable educational forum and entering its evaluation phase. New funding has been put in place to continue the efforts of the Advanced Cancer Detection Center and to transform it into a DoD Center of Excellence to recognize its capability to identify and fund outstanding research from the entire Cancer Center.

(4) **REFERENCES:** References pertinent to the individual projects are contained in the appended material.

## **APPENDIX A**

### **Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers**

**Melvyn Tockman, M.D., Ph.D.  
Robert Clark, M.D.**

# Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers (DoD Cohort Study) Progress Report

October 31, 2001

## I. Introduction

### Lung Cancer Screening with Helical CT and hnRNP A2/B1

Two promising and practical screening techniques, computerized molecular analysis of airway cell markers (ACM) and helical computed tomography (CT), are now available to examine targeted populations for the earliest signs of lung cancer. This study will compare the accuracy (sensitivity and specificity) of these screening techniques as well as the stage distribution of lung cancer detected by these methods.

Henschke et al (Lancet, 1999 354:99-105) found that 10% of helical CT-detected noncalcified nodules from 2-5 mm through 21-45 mm contained a primary lung cancer. This is four times the sensitivity of a standard chest x-ray taken at the same time. Our preliminary data (Clin Cancer Res, 1997, 3:2237-46) showed that computerized immunodetection of up-regulated hnRNP A2/B1 expression in sputum cells detected primary lung cancer in 37 of 45 (82%) cases. This is 8 times the sensitivity of standard sputum cytology obtained at the same time.

It is quite likely that the helical CT and ACM protein expression screening are complementary. The cell type distribution of the detected cancers suggests this. Henschke reported that of the 27 tumors identified by helical CT, 21 (78%) were adenocarcinomas (includes 3 bronchioloalveolar carcinomas), 3 (11%) were mixed squamous-adenocarcinoma, 1 (4%) was a squamous and 1(4%) was an atypical carcinoid. In contrast, among 13 second primary lung cancers detected by protein expression screening, 31 % were adenocarcinoma, 23% were squamous, 15% were mixed adenosquamous, 15% were small cell, 8% were large cell and 8% non-lung primary. Lung cancer cell types infrequently detected by helical CT (squamous and small cell) may be detected by ACM. Evaluation of the extent to which these early lung cancer detection techniques are complementary could only be conducted in a one-arm prospective study such as this one, where every individual is screened by all techniques at the same examination.

### Other Airway Cell Markers of Lung Cancer Evaluated with Cohort Specimens

In parallel, our NCI-EDRN Biomarker Developmental Laboratory at Moffitt (BeDLAM) grant supports the examination of other potential molecular markers on archived specimens from this Cohort trial. Several years ago with David Sidransky at Hopkins, we pioneered the use of microsatellite alterations as clonal markers in the detection of human cancer (Proc Natl Acad Sci USA 1994; 91:9871-75). We have found that microsatellite alteration and LOH on 3p is significantly associated with upregulation of hnRNP A2/B1 (Proc. AACR 1999; 40:140-1, Lung Cancer 2001; in press). Further,

loss at 3p22, the site of gene for the Type II Transforming Growth Factor Beta Receptor is strongly associated with NSCLC (Clin Cancer Res. 2001 Jun;7(6):1618-26). Altered messenger RNA and proteins of the downstream tumor suppressor TGF- $\beta$  signaling pathway are of great interest in our laboratory (Clin Cancer Res. 2001 Jun;7(6):1618-26). BeDLAM funding also supports evaluation of the extent to which gene-specific promoter hypermethylation, detected in archived sputum cells, predicts the development of lung cancer. Therefore, we have developed a technique for preservation of sputum morphology and nucleic acids so that (DNA) promoter hypermethylation and microsatellite alterations as well as altered TGF- $\beta$  type II receptor message expression (RNA markers) may be examined in the sputum specimens collected in this study.

#### *Ventilatory Obstruction (impaired spirometry) Enhances the Lung Cancer Risk of Cohort Participants*

Age and cigarette smoking are not the only lung cancer risk factors considered in this study. We have shown that current and former smokers distinguished by airways obstruction are at 2-4 fold risk of developing lung cancer compared to non-obstructed smokers (Ann Int Med 1987;106:512-8). This result has been corroborated in the Multiple Risk Factor Intervention Trial, a randomized clinical trial for the primary prevention of coronary heart disease that enrolled 12,866 men. In that study, ventilatory function was a powerful predictor of lung cancer deaths, with rates that increased from 3.02 per 1,000 person-years in the lowest quintile of forced expiratory volume to 0.43 in the highest quintile (Am J Epidemiol. 1990 Aug;132(2):265-74). A similarly high lung cancer frequency among obstructed current and former smokers has been observed more recently among the participants in the Colorado Lung Cancer SPORE (Cancer Res. 1996 Oct 15;56(20):4673-8).

#### *Former Smokers Remain at Risk of Lung Cancer and Could Benefit from Screening*

The populations of greatest interest for lung cancer screening are the estimated 46 million former smokers in the United States who remain at risk despite smoking cessation. While cardiovascular risk resolves rapidly, Wistuba et al. have shown that genetic alteration of airway lining cells observed in current smokers is not reversed in former smokers (JNCI 1997; 89:1336-73). Progression to lung cancer is probably only slowed, but not reversed by removal of the promotional stimuli of smoking. Major medical centers (Beth Israel, MD Anderson, Cancer 1996; 78:1004-10) now report similar numbers of new lung cancer cases from former as from current smokers. Former smokers, having followed the cessation advice of the medical establishment, remain at risk of lung cancer and are likely to benefit greatly from validated lung cancer screening (Cancer. 2000 Dec 1;89(11 Suppl):2506-9).

#### **Body**

##### *Start-up: Study Activation, 0-3 Months*

**Approval:** The protocol, informed consent and data collection forms were completed and this study was approved by Moffitt/USF IRB on November 5, 1998, with conditional approval by Army Regulatory Compliance on December 23, 1998. The protocol was resubmitted with amendments covering novel methods of sputum

preservation to the Moffitt/USF IRB and received Army Regulatory Compliance final approval and study activation on June 10, 1999.

*Space Renovation:* Two spirometry/sputum induction facilities were originally established. The facility at the Lifetime Cancer Screening (LCS) Center is fully operational. This facility includes a spirometry screening station, a laminar-flow sputum induction hood, and a biosafety cabinet for sputum specimen processing. Interviews and blood drawing also take place in this space. During 2001, a helical CT scanner was installed so that the entire Cohort screening process now can take place at LCS. The screening station at the James A Haley VA Hospital was used primarily for spirometry screening of volunteers identified through the VA Respiratory Division. A large number (n=1259) of Respiratory Division patients were screened and we have now exhausted that patient population for potential study participants. During 2000, the screening station at the James A Haley VA Hospital was closed.

*Equipment Purchased:* At start-up, several major pieces of equipment were purchased to support this study. These include a Helical CT scanner, a Perkin-Elmer 310 gene scanner, and an Arcturus PixCell II Laser Capture Microdissector. Several minor pieces of equipment have also been purchased, including an induction safety cabinet, nebulizer, and a sputum preparation biosafety hood. In the past year, no new equipment has been purchased for this study.

#### *Recruitment Phase, 3-24 Months*

*Staff Hired:* A total of 4.0 FTEs (2.0 FTEs funded by DoD) have been newly hired or transferred to replace the LCS staff previously working on this project. The administrative secretary and the study nurse are funded by the DoD for participant scheduling and clinical follow-up. The study coordinator and clinical research associate are funded by BeDLAM to oversee the completion of accrual, data management and specimen collection.

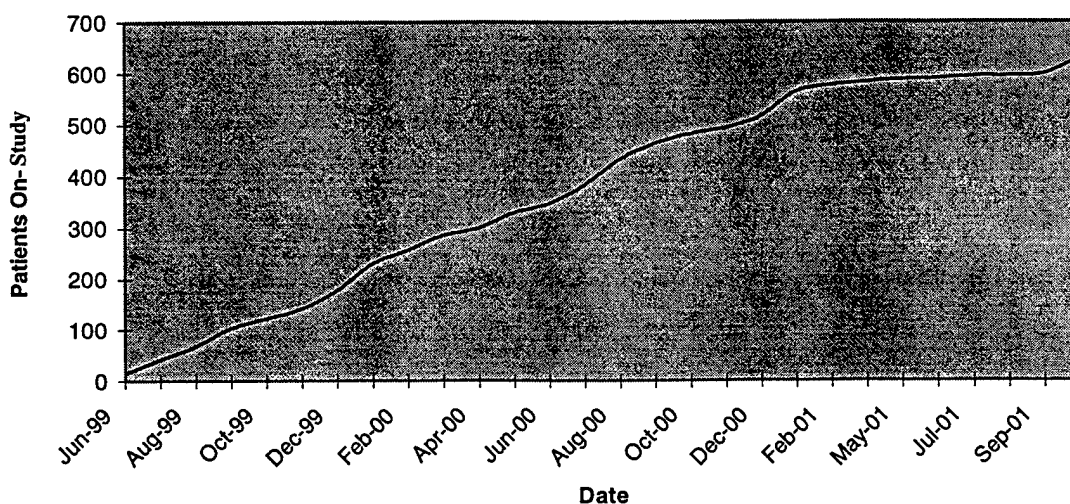
*Accrual:* The study plan provides that during the first year, 5000 subjects  $\geq 45$  years of age with  $\geq 30$  pack years of smoking would be screened by spirometry to identify 1150 subjects with mild obstruction ( $FEV_1/FVC \leq 70\%$ ). Mild obstruction would be expected to occur in 23% of these individuals. Our prior sputum/CXR screening trials have shown that in males of this age range with this smoking history, clinical lung cancer will have a 0.7% (7/1000) prevalence and 0.5% (5/1000) annual incidence. In the presence of mild obstruction, the annual lung cancer incidence increases to 1.1% (11/1000) and continues to rise with increasing obstruction. After four years of screening and depending upon the prevalence of obstruction in the study population, therefore, we had predicted 44-50 cases of lung cancer (11-13 cases per year).

At this time, 3,240 individuals have been screened, 1151 of whom were eligible to undergo spirometry. Of these, 599 (52%) met obstructive criteria and have proceeded on to sputum induction and helical CT (See chart of Cohort Accrual). Thirty-eight percent of the screened population has been referred for evaluation from the Respiratory Clinic at the James A. Haley VA hospital. This pool of recruits has more ventilatory obstruction

than the general population. Exceeding the 23% rate of mild obstruction expected from a (non-clinic) population of cigarette smokers, we find that 52% of our screened population meet the obstruction criterion. By designing the study to include younger, obstructed participants, we have indeed accrued a population at high risk for lung cancer. The observed lung cancer prevalence of 3.0% is 40% greater than expected among the 599 first examinations, and the incidence of 2.3% is 209% greater than expected among the 256 follow-up examinations. If this trend continues, more than 113 cancers would be expected to develop in this population by the end of the study, 126% greater than the required sample size.

To speed completion of accrual, therefore, the obstruction eligibility criterion was recently relaxed. Previously, to become a participant, the subject must have an  $FEV_1/FVC \leq 70\%$ . This inclusion criterion has been removed so that all levels of ventilatory function are eligible, following approval by both the University of South Florida and by DoD IRBs. Removal of the obstruction requirement has enabled 554 screened study subjects, 45 years of age and older who have smoked at least 30 pk yr, but failed the obstruction criterion to become eligible. Currently, these previously screened participants are being contacted and invited to join the study. The recent uptick in the accrual chart below reflects this procedure change. Forty unobstructed participants (included in the 1151 already screened) have newly entered active screening due to the recent change. At this rate, accrual will be complete and prevalence analysis begun by April, 2002.

**Cohort Accrual**



*Preliminary Results, Demography:* The study population currently consists of 1151 participants. From this population 599 were obstructed, are on-study and were included in the tabulation below. A total of 143 (24%) eligibles were recruited from the VA hospital. Twenty-four of 599 obstructed participants have developed lung cancer, 18 (3.0%) prevalence, 6 (2.3%) incidence. Those who developed cancer were white, 56% were male, with an average 63.2 pk yrs of smoking and an  $FEV_1/FVC$  of 60%. The age, race, and gender distributions of the cancer cases do not differ from that of the obstructed



or total screened populations (appendix 4). As might be expected, the medical histories of the obstructed population (and cancer cases) more frequently report the presence of chronic lung diseases. Preliminary comparison of occupational/environmental exposures shows no important difference (appendix 4).

*Preliminary Results, Radiographic Screening:* Two hundred and seven (34.5%) of 599 prevalence and 42 (16.4%) of 256 incidence helical CT scans have shown an abnormality (non-calcified nodule). Twenty-six (10.4%) of these lesions required diagnostic work-up due to evidence of growth on subsequent diagnostic CT scans. Of these lesions, 24 were found to be lung cancer, plus one lymphoma and one benign scar. The two most recently diagnosed (incidence) cases have not yet been staged leaving 22 for the tabulation below. Only 7 (39%) of 18 prevalence and 2 (50%) of 4 incidence lung cancers were in stage I. This stage distribution is more advanced than reported in the literature. Self-selection by symptomatic individuals is a recognized source of confounding of prevalence results. Only 5/18 (28%) of the prevalence cases and half (2/4, 50%) of the incidence cases reported symptoms (See Stage Distribution tables).

Stage Distribution of Cancer Cases								
Prevalent Cancer Cases (n=18)	NSCLC					SCLC		
	Stage 1A n=7	Stage 2B n=2	Stage 3A n=2	Stage 3B n=0	Stage 4 n=5	Limited Sm. Cell n=1	Extensive Sm. Cell n=0	Lymphoma n=1
Symptomatic n= 5								
CXR +	1	-	-	-	3	-	-	-
CXR -	-	-	-	-	1	-	-	-
Asymptomatic n= 13								
CXR +	4	1	1	-	1	-	-	-
CXR -	2	1	1	-	-	1	-	1

Incident Cancer Cases (n=4)	NSCLC					SCLC		
	Stage 1A n=2	Stage 2B n=0	Stage 3A n=0	Stage 3B n=1	Stage 4 n=0	Limited Sm. Cell n=0	Extensive Sm. Cell n=1	Lymphoma n=0
Symptomatic n= 2								
CXR +	1*	-	-	-	-	-	-	-
CXR -	-	-	-	1	-	-	-	-
Asymptomatic n= 2								
CXR +	1	-	-	-	-	-	1	-
CXR -	-	-	-	-	-	-	-	-

\*Clinical Stage Only

### Moffitt Cohort Study Results

	Study Design	Actual (as of 10/22/01)
Screened with Spirometry:	5000	1151
Eligible ~ screened with CT and Sputum:	1150 (23%)	599 (52%)
Expected Positive Prevalence Screens:	230-460 (20-40%)	245 (41%)
Expected Cancers:	50 [12-13 (1.1%)per year]	18 (3.0%) Prevalence 4 (1.56%) Incidence
Exp. Stage Distribution:	80% Stage 1	Prevalent Cases: Stage 1A: 7 (39%) Stage 2B: 2 (11%) Stage 3A: 2 (11%) Stage 3B: 0 (0%) Stage 4: 5 (28%) Limited SCLC: 1 (5.5%) Lymphoma: 1 (5.5%) Incident Cases: Stage 1A: 2 (50%) Stage 3B: 1 (25%) Extensive SCLC: 1 (25%)

*Preliminary Results, Molecular Airways Markers:* Four markers are to be assayed in the sputum of Cohort participants:

Sputum Cytology: To date 263 individual subjects have had sputum specimens processed, stained and read by a pathologist. Two (12.5%) of 16 cancer cases in the study showed sputum cytology indicative of cancer. For preliminary results see table (below).

### Cohort Sputum Cytology Results

Encounter	Number	Normal	Reg. Met	Mild Dysplasia	Mod. Dysplasia	Squamous Cancer	Adeno Cancer	Unsat.
1	184	38 (21%)	117 (64%)	14 (8%)	3 (2%)	1 (0.5%)	1 (0.5%)	10 (6%)
2	56	6 (11%)	41 (73%)	3 (5%)	N/A	N/A	N/A	6 (11%)
3	7	1 (14%)	6 (86%)	N/A	N/A	N/A	N/A	N/A
Cancer Cases	16	10 (62.5%)	1 (6.25%)	1 (6.25%)	1 (6.25%)	1 (6.25%)	1 (6.25%)	1 (6.25%)

hnRNP A2/B1 Overexpression: As outlined in the study protocol, ThinPrep monolayer slides are produced from methanol-preserved (PreservCyt) slurries of induced sputum. Following automated immunostaining (DAKO immunostainer) with monoclonal antibody 703D4 and alkaline phosphatase labeling (LSAB-II, DAKO), individual cells of interest (proplastic, metaplastic and atypical morphologies) are identified by a licensed cytotechnologist. Images of selected cells are acquired at 100 X (Nikon E800 equipped with Princeton Instruments cooled CCD) and quantified automatically for morphologic and densitometric parameters by a workstation running MetaMorph software (Universal Imaging Corp). A discriminant function developed for the Lung Cancer Early Detection Working Group (LCEDWG) Study of detection of second primary lung cancers was to be applied to distinguish positives from negatives.

The LCEDWG study has been recently concluded. Two medical oncologists (Joseph Aisner, M.D. and David Johnson, M.D.) have reviewed all cases of second primary lung cancer which serve as the "gold standard". Twenty-three second primary lung cancer cases and 20 normal controls comprised the teaching set upon which staining and scoring methods and discriminant function development were based. When applied to this same teaching set, the LCEDWG discriminant function showed a 33/43 (77%) overall accuracy. However, when applied to the LCEDWG unknown specimens, the discriminant function showed only a 35/81 (41%) accuracy. This disappointing result led to a re-evaluation of specimens that formed the basis of our 1997 published report (Clin Cancer Res, 1997, 3:2237-46). Accuracy among these specimens was 16/17 (97%), indicating reliability of assay procedures. A possible explanation for these observations comes from immunocytochemistry. Preliminary staining performed on Calu-3 cells (ATCC adenocarcinoma cell line) preserved at 4° C from 1998 to 2001 in either Saccomanno's reagent (similar to LCEDWG specimens) or DTT/EDTA followed by PreservCyt (similar to Cohort specimens) showed a significant loss of immunoreactivity in cells preserved by the method used in the LCEDWG trial (Saccomanno), compared to cells preserved by the method used in the Cohort study (PreservCyt, described above).

At present, we are resolving several issues related to immunoassay performance prior to immunostaining the Cohort specimens. In addition to resolving concerns about LCEDWG specimen preservation methods, we have been funded (Cancer Research Foundation of America) to identify the lung cancer epitope identified by NCI monoclonal antibody 703D4. Specificity of old and new lots of the 703D4 monoclonal are being compared by Western blot to 2 American and 2 Japanese antibodies against hnRNP. Epitope mapping of 703D4 has shown 3 hnRNP binding sites. Peptide oligomers with these binding site sequences have been made and used as blocking peptides in immunostaining assays. Slide preparation methods, immunostaining and quantitation are all being re-established prior to actual preparation and measurement of Cohort specimens.

Loss of heterozygosity (LOH): Sixty three alleles (Appendix III) reported in the literature to be frequently lost in NSCLC or associated with the genes for transforming growth factor  $\beta$  type II receptor (TBR II) or the downstream signaling

SMADs 2 or 4 are being examined to confirm their utility in a panel of LOH markers for Cohort specimens. After establishing the PCR conditions (using  $^{32}\text{P}$  end-labeling) for the primers at each allele, the primers are applied to archived (non-microdissected) DNA from 43 frozen paired tumor and normal samples. The frequency of microsatellite alterations (LOH or shift) are determined. The most promising markers are confirmed on tumor DNA microdissected from paraffin sections from 73 Yunnan Tin Corporation (YTC) miners who developed primary lung cancer. 9 markers from chromosomes 3p, 9p, 9q, and 17p have been selected. Primers for alleles on chromosomes 12p and 18q are being evaluated now. Once selection of the marker panel is complete, the conditions for multiplexed, automated capillary LOH assessment on the PE 310 will be established in preparation for high-throughput assay of Cohort specimens.

Promoter CpG Island Hypermethylation: Silencing of tumor suppressor genes (TSG) is one mechanism believed to underlie carcinogenesis. TSG silencing may be accomplished through gene alteration (allelic loss or gene mutation). An epigenetic mechanism, promoter CpG island hypermethylation has also been shown to silence TSG transcription (Proc Natl Acad Sci USA, 1996; 93:9821-6). Panels of primers for hypermethylation have been recently published and are under study in our laboratory. We have established the conditions for assay of p16, O<sup>6</sup>-MGMT, RAR- $\beta$ , and DAP-kinase promoter methylation in our laboratory. Four of seventeen (24%) frozen, paired (non-microdissected) DNA specimens demonstrate p16 promoter hypermethylation in our hands. To assure the quality of archived Cohort DNA, 5 Aliquots of DTT/EDTA/DMSO preserved Cohort sputum (1 cancer, 4 noncancers) have been sent to Dr. Adi Gazdar (Texas Southwestern) for assay of methylation of promoter of RASSF1, RAR- $\beta$ , p16, APC, E/H Cadherin. Preliminary results indicate the presence of satisfactorily preserved DNA at the promoter methylation sites in all specimens.

*Preliminary Results, Archive:* Nine hundred and eighty-eight induced sputum specimens (includes annual repeats) have been prepared with dithiothreitol (DTT) and EDTA, washed in Hanks solution, spun, resuspended and divided into aliquots for a) CYTYC Thin-prep slide preparation (for pap staining, immunostaining and storage), b) in CYTYC PreservCyt (methanol) slurry (at 4°C) and c) freezing with 10%DMSO/90% FBS in liquid nitrogen. Five hundred and ninety-seven blood specimens have been processed; the buffy coats have been separated and stored in liquid nitrogen. Each specimen is bar coded, and computer linked to the database of registration, demographic, medical, smoking, occupational and nutritional history data on each participant.

*Database and Lab Specimen Tracking System:* Moffitt Cancer Control Research Computing has developed an Oracle database with a Web front-end to allow registration from multiple sites. This database houses the registration, demographic, medical, smoking, occupational and nutritional history data on each participant. Since data entry is still forms-based, the data system was designed to provide easy, intelligent 'double' entry of data. The system has been programmed to provide data constraints, range and referential checks, and edit capability to keep the data clean. The data system provides tools for subject management (generate barcode labels, track unresolved data, report late forms/specimens, etc.). Finally, the relational database will easily provide data for

specific queries and statistical analysis. This Research Specimen Tracking (RST) system has now been requested for application to the NCI-SPORE-Lung Cancer Biomarker and Chemoprevention Consortium (LCBCC) study.

Moffitt Cancer Control Research Computing also has developed a Laboratory Specimen Tracking System. This study generates a large number of specimens that must undergo multiple assays in several laboratories. The Laboratory Specimen Tracking System (LST) reads the 2-D specimen barcode to log the specimen into the laboratory. The LST has been programmed to assign each type of specimen a 'profile' that specifies what will happen to the specimen in the lab. A 'profile' consists of a number of steps such as: CheckIn/CheckOut, Assay specimen acceptability, Results Reporting and Archive. The LST is able to track the progress of the specimen and let the lab manager know what step the specimen is on, the specimen turnaround time in the lab, and the archive location of the specimen and its offspring including: Slides, Sputum Slurry Bottles, and Cryovials.

### **Key Research Accomplishments**

- Developed an infrastructure to identify, accrue, screen and follow a non-diseased community-dwelling population at high risk for lung cancer.
- Developed procedures for collection and preservation of sputum specimens for new (DNA, RNA, protein and morphologic) markers of pre-neoplasia.
- Developed an archive of airways cytologic specimens suitable for evaluation of new (DNA, RNA, protein and morphologic) markers of pre-neoplasia.
- Developed an archive of white blood cells suitable to provide individual control specimens for DNA and RNA
- Developed a potency assay for MoAb 703D4 immunodetection of hnRNP A2/B1 protein expression.
- Identified a panel of 11 LOH markers for sputum lung cancer screening which identifies 84% of lung tumors (See appendix table III).

### **Reportable Outcomes**

#### Publications Related to this Study

1. Chirikos T, Hazelton T, **Tockman MS**, and Clark R. *Screening for Lung Cancer with CT: A Preliminary Cost-Effective Analysis*. CHEST. In Press.
2. Zhou J, Nong L, Wloch M, Cantor A, Mulshine JL, **Tockman MS**. *Expression of early lung cancer detection marker: hnRNP A2/B1 and its relation to microsatellite alteration in non-small cell lung cancer*. Lung Cancer. 2001. In press.

3. Mulshine JL, De Luca LM, Dedrick RL, **Tockman MS**, Webster R, Placke ME. *Considerations in developing successful, population-based molecular screening lung cancer*. Cancer. Dec 1, 2000; 89(11 Suppl):2465-7. Review.
4. **Tockman MS**, Mulshine JL. *The early detection of occult lung cancer*. Chest Surg Clin N Am. Nov 2000; 10(4):737-49.
5. **Tockman MS**. *Advances in Sputum Analysis for Screening and Early Detection of Lung Cancer*. Cancer Control, Journal of the Moffitt Cancer Center. January/February 2000, Vol. 7, No. 1:19-24.
6. Kennedy TC, Proudfoot SP, Piantadosi S, Wu L, Saccomanno G, Petty TL, **Tockman MS**. *Efficacy of Two Sputum Collection Techniques in Patients with Air Flow Obstruction*. ACTA Cytologica. July-August 1999; Vol. 43, No. 4:630-636.

#### Abstracts Related to this Study

1. Zhukov TA, Erozan YS, **Tockman MS**. *Sentinel Cell for Lung Cancer*. 14<sup>th</sup> International Congress of Cytology, RAI Congress Centre, Amsterdam, The Netherlands. May 2001.
2. Mulshine JL, **Tockman MS**, Martinez A, Man Y-G, Montuennnga L, Hong SH. *Application of Molecular Biology for Early Detection of Lung Cancer*. 1999.
3. Zhou J, Nong L, Wloch M, Zhukov TA, and **Tockman MS**. *Expression of Early Lung Cancer Detection Marker: hnRNP a2/b1 and its Relation to Microsatellite Instability in Non-Small Cell Lung Cancer*. Proc. AACR, April 1999, 40:140-141
4. Truncale T, Zhou J, Zhukov TA, Muñoz-Antonia T, Antonia S, Muro-Cacho C, **Tockman MS**. *Evaluation of TGF- $\beta$  II Receptor Expression (T $\beta$ R-II) and a Common Signaling Mediator SMAD4 in NSCLC*. Proc. AACR, April 1999, 40:337.
5. Falestiny MN, Cardona JJ, Zhou J, Zhukov TA, Nong L, Solomon DA, **Tockman MS**. *Transforming Growth Factor  $\beta$  Type II Receptor Expression in Non-Small Cell Lung Cancer, Viral Transformed Bronchial Cells and Normal Bronchial Epithelial Cells: A Comparative Study*. Chest (Suppl); November 1998.
6. **Tockman MS**, Saccomanno G, Michels R, Zhukov TA, Erozan Y, and Gupta P. *Sentinel Cell for Lung Cancer*. 6<sup>th</sup> SPORE Investigator's Workshop; July 1998.

#### Presentations Related to this Study

December 8-10, 1998 *International Conference on Prevention and Early Diagnosis of Lung Cancer, Johns Hopkins Lung Project and Immunocytochemical Screening for Lung Cancer*. University of Varese and University of Massachusetts Medical School, Varese, Italy.

- February 12, 1999 ***ALCASE Workshop – Lung Cancer: A Revolution in Care, Technology in Early Diagnosis of Lung Cancer.*** Embassy Suites, Tampa, Florida
- April 26, 1999 ***1999 ALA/ATS International Conference Program, Early Sputum Marker for Lung Cancer (hnRNP).*** San Diego Convention Center, San Diego, California
- April 30, 1999 ***Pharmacology Seminar Program, Detection and Immunostaining of the Lung Cancer Sentinel Cell.*** University of Pittsburgh, Pennsylvania.
- September 13, 1999 ***Advanced Cancer Detection Center, External Advisory Committee.*** Moffitt Cancer Center, Tampa, Florida
- September 30<sup>th</sup> to October 3, 1999 ***The First International Conference On Screening for Lung Cancer,*** Cornell University, New York
- October 9-13, 1999 ***Annual Congress of the European Respiratory Society, Dysregulation of the Cell Cycle in Lung Cancer.*** Madrid, Spain
- October 15-16, 1999 ***Molecular Biomarkers Workshop,*** Roy Castle Lung Cancer Foundation, Liverpool, England
- October 26, 1999 ***Screening of Lung Cancer Conference,*** Gaithersburg, MD
- October 31, 1999 ***7<sup>th</sup> Annual Scientific Assembly of the American Association of Bronchology, New horizons in cytological based early detection in lung cancer.*** Chicago, IL
- February 9, 2000 ***International Symposium on Early Detection of Lung Cancer, Molecular Screening Program: Past, Present, and Future.*** Tel Aviv, Israel
- February 27-29, 2000 ***International Agency for Research on Cancer, Use of Biomarkers in Chemoprevention of Cancer, Lung Cancer: Intermediate Effect Markers.*** Heidelberg, Germany

March 20, 2000	<i>Cahan Lectureship at Memorial-Sloan Kettering, Molecular Screening for Lung Cancer. New York, NY</i>
April 12, 2000	<i>Early Detection Research Network Site Visit at H. Lee Moffitt Cancer Center &amp; Research Institute, Organization of BeDLAM. Tampa, FL</i>
June 16, 2000	<i>Wayne State University Cancer Conference, Sputum in 2000: Hypothetical Advantages, Practical Limitations, and Novel Approaches, Detroit, MI</i>
June 22, 2000	<i>Reducing Lung Cancer Mortality: Actions for the New Millenium, Sputum Based Detection of Preinvasive Lung Cancer, Washington, DC</i>
June 27, 2000	<i>Roy Castle Lung Cancer Foundation and H. Lee Moffitt Cancer Center, Quest for the Cure, Lung Cancer Screening and Early Detection: Spiral CT Scanning and Molecular Markers, Tampa, FL</i>
July 19, 2000	<i>H. Lee Moffitt Cancer Center/USF Lung Cancer Conference, Epidemiology and Early Detection of Lung Cancer, Coeur d'Alene, ID</i>
July 19, 2000	<i>H. Lee Moffitt Cancer Center/USF Lung Cancer Conference, The Management of Pre-Clinical Lung Cancer, Coeur d'Alene, ID</i>
September 12, 2000	<i>IASLC 9<sup>th</sup> World Conference on Lung Cancer, Cellular Targeting in the Molecular Diagnosis of Lung Cancer, Tokyo, Japan</i>
October 24, 2000	<i>66<sup>th</sup> International Scientific Assembly of the ACCP, San Francisco, CA</i>
	<i>ACCP Post Graduate Course, Screening and Early Detection of Lung Cancer</i>
	<i>Meet the Professor, Sputum Detection of Early Lung Cancer: Hypothetical Advantages, Practical Limitations, and Novel Approaches</i>
October 27, 2000	<i>Cornell CT Conference, Sputum Detection of Early Lung Cancer: A Compliment to Helical CT, New York, NY</i>



- March 7-8, 2001     ***Lung Cancer Early Detection Workshop***, National Cancer Institute/ American Cancer Society, "***New Frontiers of Screening Science***". Rockville, MD
- March 24-25, 2001     ***Second Annual – A Practical Pulmonary Review for Primary Care Providers***, University of South Florida/Department of Veterans Affairs, James Haley, "***Early Recognition of Lung Cancer***". St. Pete Beach, FL
- June 20-22, 2001     ***Early Detection Research Network***, National Institute of Health/National Cancer Institute, "***Lung Cancer Screening Update***" and "***Industrial Partnership with EDRN***", Washington, DC
- June 26-July 2, 2001     ***Second International Lung Cancer Molecular Biomarkers Workshop "A European Strategy for Developing Lung Cancer Molecular and Clinical Diagnostics in High Risk Populations"***, Roy Castle Lung Cancer Foundation, "***Sputum in 2001: Hypothetical Advantage Practical Limitations, and Novel Approaches***" and "***Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers***" Liverpool, England
- August 7-12, 2001     ***3<sup>rd</sup> International Conference on Prevention & Early Detection of Lung Cancer***, International Association for the Study of Lung Cancer, "***Cellular Approaches to Lung Cancer Detection***" and "***Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers***". Reykjavik, Iceland

The first International Conference on Lung Cancer Screening was held at Cornell in October 1999. A group of international experts in imaging, molecular diagnostics, pulmonology, oncology, epidemiology, clinical trial design, statistics, health care policy and patient advocacy met to address the issues central to lung cancer screening. The meeting was co-sponsored by the ACS, the National Cancer Institute, ALCASE, Weill Medical College of Cornell University and other organizations. The conference reviewed currently available data on lung cancer screening and engaged in intensive analyses of the implications with a view to attaining consensus with respect to the main issues surrounding early detection of lung cancer. The conference organizers asked for two reports of this study "Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers." Dr. Robert Clark, Moffitt Director of Radiology described lung cancer screening with helical CT, while Dr. Tockman presented the Moffitt experience with protein expression screening of ACM.

The Moffitt trial comparing ACM and helical CT was recognized as one of three such studies in the nation that was capable of providing insight into definitive clinical

trial design considerations. ("The sites at which CT screening is currently being performed in the United States are Weill Medical College of Cornell University, New York NY; H. Lee Moffitt Cancer Center, Tampa FL; Mayo Clinic, Rochester MN." ). The conference appreciated that to answer certain questions, an unscreened comparison group was needed to supplement the three ongoing "one-armed" trials. The conference concluded, "comparative populations could be constructed by matching cases (e.g., age, smoking history, tumor classification) from populations currently enrolled in existing large studies or databases (e.g., PLCO screening trial, SEER). Such new methodologic approaches in response to a perceived public health emergency (i.e., 85% lung cancer mortality) may constitute an important precedent for public health research. The results of this innovative approach may guide public policy in formulating lung cancer screening recommendations and save a significant number of lives. As such, these activities merit high priority for creative funding support."

The complete text of the Consensus Statement from the First International Conference on Screening for Lung Cancer may be found in Appendix I of this report.

#### **Funding Received Based Upon Work Supported by this Award**

1. "The Biomarker Development Laboratory at Moffitt" (NCI-CA 84973, M. Tockman, PI, 1<sup>st</sup> year/Total award \$413,720/\$1,903,827).
2. "Identification of the lung cancer epitope identified by the monoclonal antibody 703D4" (Cancer Research Foundation of America, M. Gruidl, PI, Total award \$38,950)
3. J. Park, PI, NCI-EDRN, Total award \$98,000.

#### **Conclusion**

The Markers of Transformation in Airways Epithelial Cells from a Cohort of Obstructed Smokers and Former Smokers (DoD Cohort Study) performs two exciting and valuable functions for the Moffitt Advanced Cancer Detection Center. First, it is the only study in the nation that currently evaluates both molecular airways markers and helical CT examinations simultaneously in the prospective detection of lung cancer. Until the Cornell and Mayo studies begin their collection of sputum specimens, no other study addresses the relative merits of these apparently complementary techniques for lung cancer screening. This research question addresses the most common cause of cancer death and the only common cancer for which no screening is available. Second, the archive of radiographs, sputum and blood cell specimens provides an infrastructure for other investigators at Moffitt and across the nation. The recent award to Dr. Jong Park of an NCI Early Detection Research Network grant was based on the availability of the Cohort archive. Similarly, the collaboration with SPORE investigators at Texas Southwestern (Drs. Gazdar and Minna) and at Johns Hopkins (Drs. Baylin and Herman) are based upon the availability of Cohort specimens. The prospective design, innovative methods and careful execution of this study make it a valuable scientific contribution.

## Appendix I

### THE FIRST INTERNATIONAL CONFERENCE ON SCREENING FOR LUNG CANCER

October 1 - 3, 1999

Weill Medical College of Cornell University, New York, NY  
Consensus Statement

#### Summary

Lung cancer kills more individuals than cancers of the breast, colon, cervix and prostate combined. Recent scientific advances create an extraordinary potential to develop a lung cancer screening program that would prevent untimely deaths of vast numbers of current and former smokers who remain at high risk despite smoking cessation. The most promising of these scientific advances are rapid (single breath-hold helical) CT and computerized molecular analysis of airway cell markers (ACM). Each identifies lung cancers much earlier in their development than previously possible with conventional techniques and are likely to be complementary to each other in enhancing early detection of lung cancer. There is compelling evidence that use of these approaches can lead to high cure rates of lung cancer, a disease which currently has a dismal outcome.

The technology to implement these screening approaches currently exists and could rapidly be extended to offer screening to high-risk populations of smokers and former smokers. This creates an urgent need for research to evaluate how these techniques can best be utilized and the magnitude of the benefit they can create in order to allow appropriate public policy decisions about screening for lung cancer.

In December 1998, the International Conference on Prevention and Early Diagnosis of Lung Cancer, sponsored by the American Cancer Society (ACS), the Union International Contre Le Cancer (UICC), the Alliance for Lung Cancer Advocacy, Support, and Education (ALCASE) and other international organizations re-examined the recommendations against screening for lung cancer. The experts concluded that the information leading to these recommendations based on previous screening trials conducted more than two decades ago had a number of limitations and thus comprised an imperfect basis for current health policy. In light of emerging information and the enormous importance of the lung cancer problem, that conference recommended urgent reconsideration of issues surrounding early detection of lung cancer.

In response to this challenge, the first International Conference on Lung Cancer Screening was held in October 1999. A group of international experts in imaging, molecular diagnostics, pulmonology, oncology, epidemiology, clinical trial design, statistics, health care policy and patient advocacy met to address the issues central to lung cancer screening. The meeting was co-sponsored by the ACS, the National Cancer Institute, ALCASE, Weill Medical College of Cornell University and other organizations. The conference reviewed currently available data on lung cancer screening and engaged in intensive analyses of the implications with a view to attaining consensus with respect to the main issues surrounding early detection of lung cancer.

It was agreed that subsequent to the institutional policy statements not recommending screening for lung cancer, two important developments have occurred. Compelling evidence has continued to emerge over the past decades that resection of early lung cancer has major bearing on survival, and new techniques now provide for distinctly earlier detection of the disease. From this it follows that modern screening for lung cancer would save lives. Beyond this qualitative conclusion, there is an urgent need to learn about the magnitude of this effect.

It was recognized that more than 20,000 people have already participated in studies evaluating the efficacy of CT screening for lung cancer in the United States, Europe, Middle East, and Japan and more than 9000 using ACM. It was agreed that to further quantify the magnitude of the effect, the recommendation to use randomized controlled trials requires serious reconsideration for several reasons. The principal reasons among these are the high cost, long duration of such studies and the rapid advances in technology, together with existing visions of a less expensive and more rapid approach, one already being implemented by ongoing studies.

The conference agreed to form working groups and on a need to reconvene within six months to more closely review the ongoing studies and other interim developments.

#### Expanded statement

Screening test(s) for lung cancer should be simple, inexpensive, noninvasive, and potentially widely available with a demonstrated acceptable level of sensitivity, specificity, and predictive value. Computed tomography and automated airway cell marker analysis were considered the most promising as well as being complementary (e.g., with regard to detection of central versus peripheral tumors and squamous versus adenocarcinoma histology). Other modalities were also considered but were deemed to either not meet the requirements stated above as they were still too early in their investigative course or more appropriate for diagnostic evaluation. These included: chest radiography (plain; digital, with or without computer-assisted diagnosis; energy-subtraction imaging, with or without such diagnostics), positron emission tomography, electrical impedance tomography imaging, magnetic resonance imaging, fluorescent light bronchoscopy, and CT virtual bronchoscopy.

Screening is only of value when it is linked with appropriate diagnostic interventions and treatment. The conference attendees concluded further evaluations of lung cancer screening should be conducted within the framework of an overall research program. Such a program would include standardization of diagnostic evaluation and treatment to minimize unnecessary diagnostics, invasive procedures and surgery. Further evaluation of treatment interventions should also be considered. It was decided that all future screening evaluation should be performed in a programmatic setting which includes outcome evaluation, quality assurance, standardized interpretation, diagnostic evaluation, organized reporting and results communications and education for physicians and screeners.

Very promising data from CT screening trials of about 20,000 screening subjects worldwide were presented, underscoring the need to evaluate these tools rapidly. To permit rapid evaluation, an infrastructure must be developed to allow for assessment of future screening modalities. Essential first steps include standardizing protocols, pooling of cohort data, and identifying support mechanisms to ensure long-term clinical follow-up of vanguard populations. **The sites at which CT screening is currently being performed in the United States are Weill Medical College of Cornell University, New York NY; H. Lee Moffitt Cancer Center, Tampa FL; Mayo Clinic, Rochester MN.** Other centers abroad are Muenster University, Muenster, Germany; Hadassah Medical Center, Jerusalem, Israel; the National Cancer Center Hospital, Tokyo, Japan; and Shinshu University, Japan. It was also suggested that additional sites be added in an organized fashion to allow the rapid collection of sufficient screening data to refine and recommend definitive clinical trial design considerations. **To supplement this information, comparative populations could be constructed by matching cases (e.g., age, smoking history, tumor classification) from populations currently enrolled in existing large studies or databases (e.g., PLCO screening trial, SEER). Such new methodologic approaches in response to a perceived public health emergency may constitute an important precedent for public health research. The results of this innovative approach may guide public policy in formulating lung cancer screening recommendations and save a significant number of lives. As such, these activities merit high priority for creative funding support.**

Further research is necessary to determine optimum details for the target population characteristics (age, smoking history, etc.), periodicity of screening, noninvasive diagnostic algorithms after abnormal screening results, and invasive tissue sampling and treatment algorithms. In the conduct of these studies, consideration should be made to bank (store) images as well as certain samples from screened subjects for further study (e.g., sputum, blood, and exfoliated oral cells).

Three possible evaluation strategies were discussed in detail including the strengths, limitations, and implications of a choice of one design compared with the others. The currently recommended entry criteria for enrollment are: current or former (less than ten years since quitting) smokers, age 50 or more, and healthy enough to withstand thoracotomy (as determined by pulmonary function test). Such entry criteria are critical since an extension to other population groups may require a new trial according to the orthodox view.

The randomized clinical trial (RCT) with lung cancer mortality as its endpoint is the design that offers protection against the unknown influence of suspected biases such as selection bias, lead-time bias, length bias sampling, and over-diagnosis. It was recognized that such a trial could require 80,000 individuals to be randomized to an active population (AP) or a control group (passive population (PP)). A baseline screening and four annual repeat screenings with a follow-up period of 8 years after the last screen would be performed. Earliest opportunity for definitive data from such a trial, if it were to begin in 2000, is between 2009 and 2013 (a total of at least 3 rounds with a minimum of 5 years of follow-up). The large sample size is based on an unanticipated, but possibly

worst case scenario of low benefit (10% mortality reduction) and high contamination in the PP.

The benefit of such a design is that the intervention would be evaluated without the influence of unknown biases. However, its high financial cost could lead to greater compromises (e.g., unknown threats to power due to erosion of the integrity of the randomization over time, in particular contamination; lack of acceptance of the study conclusion; new technology overtaking the technology under evaluation, a potential lack of participants because of publicity about the presumed superior efficacy of CT).

A RCT using a surrogate endpoint still provides protection against selection bias but provides an earlier answer at lower costs. The surrogate must be a direct goal of screening (e.g., a more favorable stage-shift (TNM), tumor size, % positive nodes, histology) and strongly predictive of mortality. In such a trial, 40,000 subjects would be required. A baseline and four annual repeat screenings would be required with a follow-up period of at least 3 years after the last screen. The earliest opportunity for definitive data, if the trial were to begin in 2000, would be between 2005 and 2008. While issues related to access to control-group endpoints would need to be resolved the benefits of the design include lower cost and more rapidly available data regarding the question of test efficacy. The concern about this approach includes the significant investment in time, the magnitude of potential error which may be difficult to quantify, lack of consensus about interpretation of end results-some policy makers may not accept the end result based on an approach that depends on predictors of mortality.

The third design, the non-comparative or quasi-comparative design, would require 8,000 to 10,000 individuals. It would require a baseline and a single annual repeat. Follow-up for both surrogate and mortality endpoints would be done, but only on the estimated 300 to 400 malignancies. The earliest opportunity for definitive data, if the study were to begin in 2000, would be 2002. Issues to be addressed would include access to comparison groups (PLCO screening study or older studies) and matching by relevant factors (e.g., histology, age, smoking history). The benefit of this design is that the data would be available more quickly and be obtained at a lower cost.

In summary, it was concluded that:

Ø A RCT with a death endpoint offers the most unbiased answer, but is not embraced with enthusiasm due to costs (mostly time) and the realistic appraisal that precision erodes over time. In some countries, a decision to offer lung cancer screening may require results from a RCT with a mortality endpoint.

Ø A RCT with surrogate endpoints is more attractive because of its inherent economy. Workgroup members were troubled by uncertainty that the intermediate endpoints accurately indicated mortality. It was also recognized that if one is willing to accept a study with surrogate endpoints, one should be able to accept a non-comparative study.

ØAt a minimum, the concept that funding for multiple designs should be considered rather than relying only on a single strategy to evaluate lung cancer screening. Further, the urgency of the public health problem warrants an immediate response by health agencies and professional organizations to support organized data collection and evaluation of the potential benefit and costs of all study designs to answer important questions.

ØA non-comparative design could be used, following similar selection criteria. Data should be accumulated from the international sites currently performing helical CT studies and molecular analysis of airway cell markers. Common data collection procedures for all centers should be organized. This effort should be multi-disciplinary in order to measure all end-results (detection data and follow-up) and address harms as well as benefits, including psycho social issues. Models for efficacy and cost-effectiveness of surrogate endpoint and non-comparative designs should be developed. Efforts should be made to seek to answer questions about selection effect, lead time bias, length bias sampling (and overdiagnosis). Realistic estimates of the influence of potential biasing factors may result in greater applicability of alternative designs using comparative data (e.g., National Cancer Institute trial data such as PLCO, SEER registry, etc.)

It was felt that it was important to seek leadership from the appropriate specialty organizations (e.g., American College of Radiology, American College of Pathology, American Thoracic Society) to insure quality assurance for helical CT and computerized molecular analysis of airway cell markers, guidelines for these tests, subsequent interventions, and pathology

Evaluation of screening for lung cancer is critically dependent on accurate pathologic diagnosis of the disease. The majority of CT-detected malignancies are peripheral adenocarcinomas and, thus, patient specimens will include putative precursor lesions (i.e., pre-invasive lesions, solitary non-invasive non-mucinous bronchioloalveolar carcinomas and small invasive adenocarcinomas as well as occasional central airway squamous lesions). While not all cytologists and surgical pathologists are familiar with the current 1999 WHO/IASLC classification of lung tumors, the diagnosis of lung cancer is made with a high degree of accuracy. Difficulties in diagnosis are most often related to tumor sampling, the size of the sample and artifacts. The concept of overdiagnosis should not be confused with a false positive diagnosis of lung cancer by pathologists as these are, fortunately, exceedingly rare.

The early detection of lung cancer by helical CT and computerized molecular analysis of airway cell markers provides an important opportunity for radiologic-pathologic and clinical correlation. Since little is known about the clinical course of atypical adenomatous hyperplasia, solitary non-invasive non-mucinous bronchioloalveolar carcinoma, and early phase invasive adenocarcinomas, a single protocol for specimen handling and a central tissue registry are essential. An international panel will be utilized to reach consensus on difficult lesions, and the tissue bank will insure further clinical, radiographic, light microscopic, immunohistochemical and molecular studies of putative

precursor lesions and small carcinomas. Through the collection of these lesions we can further our understanding of the biologic behavior of lung cancer.



## **Appendix II**

### **Moffitt Patient Recruitment Work Summation**

#### **Letter writing**

A total of eight letters were developed. Each letter was specific for the following target audience:

- Mayor Dick Greco
- County administrator Daniel Kleman
- Benevolent organization (director, administrator, commander)
- Benevolent organization members
- Major employers
- Employees of major employers
- City employees
- County employees

#### **Contacts**

**Benevolent organizations**

Names, addresses, and phone numbers were generated for twenty-nine benevolent organizations in the Tampa/Hillsborough county area.

**Major employers**

Names, addresses, and phone numbers were generated for the top sixteen employers (total employees) in the Tampa/Hillsborough county area.

Minimal Internet research completed for current lung cancer figures and address for county administrator Daniel Kleman.

#### **Communications**

The most significant portion of work hours involved calling the specific organizations/companies and locating the appropriate person with the authority to review the materials and grant permission for distribution to members/employees.

**Script development**

A script was developed for phone contact with organizations to ensure the recipient received a consistent persuasive message. No organizations or companies contacted refused the opportunity to review the recruitment materials.

A list of interested benevolent organizations and major employers was sent to Dr. Tockman.

#### **Lifetime Cancer Screening Pamphlet**

Suggestions for revisions to the pamphlet were reviewed with Dr. Teri Albrecht, a specialist in research communication from the USF College of Public Health.

# Appendix III

## Identification of Panel of Primers for Cohort Loss of Heterozygosity (LOH) Determination

Name	Location	Adjacent gene	Learning Samples Completed	Results (% with MA)	Test Samples Completed	Results (% with MA)
D3S1007	3p25	NA	43 paired tumor and normal samples	27.9	73 YTC tumor samples	28.77
D3S1067	p21.1-14.3	NA	43 paired tumor and normal samples	22.3	73 YTC tumor samples	31.51
D3S1284	p13-12	NA	43 paired tumor and normal samples	9.3	73 YTC tumor samples	10.96
ACTBP2	5p-q	NA	43 paired tumor and normal samples	25.58		
D8S348	q24.13-24.3	NA	43 paired tumor and normal samples	11.63		
D8S320	8p-q	NA	43 paired tumor and normal samples	4.65		
D9S171	9p21	p16	43 paired tumor and normal samples	18.6	73 YTC tumor samples	15.07
D9S242	q32-33	NA	43 paired tumor and normal samples	11.62	73 YTC tumor samples	35.62
D9S753	q22.1-22.3	P16/IFNA?	43 paired tumor and normal samples	9.3		
IFNA	9p22	IFNA	43 paired tumor and normal samples	20.93	73 YTC tumor samples	25.4
D11S488	q24.1-25	NA	43 paired tumor and normal samples	4.65		
D20S82	20p-q	NA	43 paired tumor and normal samples	9.3		
D20D85	20p-q	NA	43 paired tumor and normal samples	13.95		
D17S122	17P12-11.2	NA			73 YTC tumor samples	23.28
CHRNA1	17P12-11	cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)			73 YTC tumor samples	15.1
TP53	17P13.1	TP53			73 YTC tumor samples	26.02
D3S1260	3P23-21	TBR II	19 paired tumor and normal samples	5		
D3S1537	3P24.2-22	TBR II	19 paired tumor and normal samples	26		
D3S2317	3P24.2-22	TBR II	19 paired tumor and normal samples	5		
D3S647	3P24.2-22	TBR II	19 paired tumor and normal samples	10.5		
D3S1211	3P24.2-22	TBR II	19 paired tumor and normal samples	15.8		
D3S1483	3P24.2-22	TBR II	19 paired tumor and normal samples	15.8		
D3S2319	3P23-21	TBR II	19 paired tumor and normal samples	15.8		
D3S1100	3P22-21	TBR II	19 paired tumor and normal samples	15.8		
D3S1283	3P24.2-22	TBR II	19 paired tumor and normal samples	26		
D3S1583	3P24.2-22	TBR II	19 paired tumor and normal samples	21		
D3S1619	3P24.2-22	TBR II	19 paired tumor and normal samples	31.6		
D3S1266	3P24-23	TBR II	19 paired tumor and normal samples	5		
D3S1227	3P24.2-22	TBR II	19 paired tumor and normal samples	15.8		

Name	Location	Adjacent gene	Status of research-1	Results (% with MA)	Status of research-2	Results (% with MA)
D3S1612	3P24.2-22	TBR II	19 paired tumor and normal samples	21.1		
D3S1609	3P24.2-22	TBR II	19 paired tumor and normal samples	10.5		
D3S1611	3P24.2-22	TBR II	19 paired tumor and normal samples	10.5		
D3S1448	3P22-21	TBR II	19 paired tumor and normal samples	5		
D3S1449	3P22-21	TBR II	19 paired tumor and normal samples	NA		
D3S1298	3P24.2-22	TBR II	19 paired tumor and normal samples	15.8		
D12S99	12p13	CDKN1B	Defining PCR conditions			
D12S320	12p13	CDKN1B	PCR conditions established			
D12S269	12p13	CDKN1B	Defining PCR conditions			
D12S77	12p13	CDKN1B	Defining PCR conditions			
D12S358	12p13	CDKN1B	Defining PCR conditions			
D12S1697	12p13	CDKN1B	PCR conditions established			
D12S98	12p13	CDKN1B	PCR conditions established			
D12S89	12p13	CDKN1B	PCR conditions established			
D12S391	12p13	CDKN1B	PCR conditions established			
D18S535	18q12.3	NA	PCR conditions established			
D18S454	18q12.3-q21.1	NA	Defining PCR conditions			
D18S460	18q21	SMAD2	PCR conditions established			
D18S1118	18q21	SMAD2	PCR conditions established			
D18S450	18q21.1	SMAD2	Defining PCR conditions			
D18S470	18q21.1	SMAD2	PCR conditions established			
D18S474	18q21.1	SMAD2/SMAD4	Defining PCR conditions			
D18S1126	18q21.1	SMAD4	Defining PCR conditions			
D18S1110	18q21.1	SMAD4	Defining PCR conditions			
D18S1099	18q21.1	SMAD4	PCR conditions established			
D18S845	18q21.1	SMAD4	Defining PCR conditions			
D18S46	18q21.1	SMAD4/DCC	Defining PCR conditions			
D18S484	18q21.1	DCC	PCR conditions established			
D18S363	18q21.1	DCC	PCR conditions established			
D18S851	18q21.1	DCC	PCR conditions established			
D18S35	18q21.1	DCC	PCR conditions established			
D18S487	18q21.1	DCC	PCR conditions established			
D18S858	18q21.2	DCC	Defining PCR conditions			
D18S64	18q21.1-32	DCC	PCR conditions established			

Appendix IV  
Preliminary Results

**Preliminary Demographic Results**

	<b>Total Population</b>	<b>Obstructed</b>	<b>Cancer</b>
<b>N</b>	<b>1150</b>	<b>596</b>	<b>22</b>
<b>Age</b>	<b>60.7</b>	<b>62.5</b>	<b>63.2</b>
<b>Race, white</b>	<b>95.9</b>	<b>95.1</b>	<b>100</b>
<b>Gender, male</b>	<b>60.0</b>	<b>61.1</b>	<b>55.6</b>
<b>Smoking, p/y</b>	<b>58.1</b>	<b>61.8</b>	<b>63.2</b>
<b>FEV/FVC</b>	<b>66.3</b>	<b>56.7</b>	<b>60.1</b>

# Appendix IV Preliminary Results

Cohort Statistics for All Subjects N=1151

Pack Year	#Subject	%Subjects	%FEV	#Subjects	%Subjects	Age	#Subjects	%Subjects	Gender	#Subjects	%Subjects	Race	#Subjects	%Subjects
<30	3	0.26%	<20	0	0.00%	45-49	97	8.43%	Female	467	40.57%	African American	16	1.39%
30-34	131	11.38%	20-24	2	0.17%	50-54	199	17.29%	Male	689	59.86%	African Amer Hispanic	0	0.00%
34-39	137	11.90%	25-29	7	0.61%	55-59	243	21.11%				Amer Indian, Eskimo	5	0.43%
40-44	135	11.73%	30-34	20	1.74%	60-64	234	20.33%				Eastern Indian Amer	1	0.09%
45-49	136	11.82%	35-39	26	2.26%	65-69	202	17.55%				Asian or Pacific Islander	1	0.09%
50-54	107	9.30%	40-44	30	2.61%	70-74	118	10.25%				White non-Hispanic	1109	96.35%
55-59	72	6.26%	45-49	49	4.26%	75-79	54	4.69%				White Hispanic	21	1.82%
60-64	86	7.47%	50-54	72	6.26%	80-84	9	0.78%				Other	3	0.26%
65-69	47	4.08%	55-59	80	6.95%	85-89	0	0.00%						
70-74	50	4.34%	60-64	138	11.99%									
75-79	56	4.87%	65-69	175	15.20%									
80-84	41	3.56%	70-74	203	17.64%									
85-89	19	1.65%	75-79	204	17.72%									
90-94	33	2.87%	80-84	113	9.82%									
95-99	17	1.48%	85-89	28	2.43%									
100-110	40	3.48%	90-94	2	0.17%									
111-120	15	1.30%	95-99	0	0.00%									
121-130	6	0.52%												
131-140	11	0.96%												
141-150	3	0.26%												
151-160	1	0.09%												
161-170	1	0.09%												
171-180	1	0.09%												
181-190	1	0.09%												
191-200	1	0.09%												
>200	1	0.09%												

Cohort Statistics for All Patients N=1151

Family Cancer	# Subjects	% Subjects	Disease	# Subjects	% Subjects	Material	#Subjects	% Subjects	Industry	# Subjects	% Subjects
Family Cancers	805	69.94%	Cancer	48	4.17%	Coal	275	23.89%	UranMine	11	0.96%
			Asthma	128	11.12%	Dyes	121	10.51%	OpenPitMine	34	2.95%
			Asbestos	19	1.65%	Radon	43	3.74%	UndgrdCoalMine	15	1.30%
			Emphysema	157	13.64%	Nickel	31	2.69%	UndgrdHardRockMin	16	1.39%
			Tuberculosis	24	2.09%	Arsenic	31	2.69%	Smelting	29	2.52%
			Glaucoma	46	4.00%	Asbestos	257	22.33%	IronSteelMan	65	5.65%
			Hypertension	107	9.30%	Beryllium	12	1.04%	FoundryWork	62	5.39%
			Heart Arrythmia	85	7.38%	Chromium	32	2.78%	ChemPetro	104	9.04%
			Heart Attack	93	8.08%	Iron Ore	59	5.13%	AutoRepair	151	13.12%
			Chronic Bronchitis	157	13.64%	Silica Dust	87	7.56%	PipeFitting	68	5.91%
			COLD COPD	107	9.30%	Formaldehyde	153	13.29%	PipeCover	79	6.86%
			Other Chronic Lung	29	2.52%	Gas Petro	416	36.14%	BuildInsulation	134	11.64%
			Pneumonia	147	12.77%	X-ray/Rad	510	44.31%	BuildDemo	85	7.38%
						Metal Process	129	11.21%	Welding	126	10.95%
						Pesticide/Herbicide	492	42.75%	Sandblasting	72	6.26%
						BisEther	114	9.90%	Fishing	20	1.74%
									Farming	176	15.29%
									LumberPaper	104	9.04%
									BoatBuild	67	5.82%
									LeatherWork	23	2.00%
									PeelCleanBoilers	51	4.43%

Cohort Statistics for Abnormal Spirometry (Obstructed Patients) Subjects Only N=599

Pack Year	#Subject	%Subjects	%FEV	#Subjects	%Subjects	Age	#Subjects	%Subjects	Gender	#Subjects	%Subjects	Race	#Subjects	%Subjects
<30	2	0.33%	<20	0	0.00%	45-49	34	5.68%	Female	237	39.57%	African American	14	2.34%
30-34	61	10.18%	20-24	2	0.33%	50-54	80	13.36%	Male	362	60.43%	African Amer Hispanic	0	0.00%
34-39	43	7.18%	25-29	7	1.17%	55-59	110	18.36%				Amer Indian, Eskimo	3	0.50%
40-44	67	11.19%	30-34	20	3.34%	60-64	128	21.37%				Eastern Indian Amer	1	0.17%
45-49	62	10.35%	35-39	26	4.34%	65-69	115	19.20%				Asian or Pacific Islander	1	0.17%
50-54	59	9.85%	40-44	30	5.01%	70-74	86	14.36%				White non-Hispanic	570	95.16%
55-59	39	6.51%	45-49	49	8.18%	75-79	39	6.51%				White Hispanic	9	1.50%
60-64	47	7.85%	50-54	72	12.02%	80-84	7	1.17%				Other	1	0.17%
65-69	24	4.01%	55-59	80	13.36%	85-89	0	0.00%						
70-74	31	5.18%	60-64	138	23.04%									
75-79	40	6.68%	65-69	175	29.22%									
80-84	25	4.17%	70-74	0	0.00%									
85-89	12	2.00%	75-79	0	0.00%									
90-94	22	3.67%	80-84	0	0.00%									
95-99	12	2.00%	85-89	0	0.00%									
100-110	26	4.34%	90-94	0	0.00%									
111-120	10	1.67%	95-99	0	0.00%									
121-130	3	0.50%												
131-140	6	1.00%												
141-150	2	0.33%												
151-160	0	0.00%												
161-170	0	0.00%												
171-180	1	0.17%												
181-190	1	0.17%												
191-200	1	0.17%												
>200	1	0.17%												

Cohort Statistics for Patients with Abnormal Spirometry N=599

Family Cancer	# Subjects	% Subjects	Disease	# Subjects	% Subjects	Material	#Subjects	% Subjects	Industry	# Subjects	% Subjects
Family Cancers	405	67.61%	Cancer	22	3.67%	Coal	156	26.04%	UranMine	5	0.83%
			Asthma	86	14.36%	Dyes	60	10.02%	OpenPitMine	24	4.01%
			Asbestos	13	2.17%	Radon	25	4.17%	UndgrdCoalMine	9	1.50%
			Emphysema	131	21.87%	Nickel	20	3.34%	UndgrdHardRockMin	8	1.34%
			Tuberculosis	14	2.34%	Arsenic	23	3.84%	Smelting	15	2.50%
			Glaucoma	28	4.67%	Asbestos	144	24.04%	IronSteelMan	39	6.51%
			Hypertension	57	9.52%	Beryllium	7	1.17%	FoundryWork	35	5.84%
			Heart Arrythmia	48	8.01%	Chromium	19	3.17%	ChemPetro	59	9.85%
			Heart Attack	51	8.51%	Iron Ore	35	5.84%	AutoRepair	81	13.52%
			Chronic Bronchitis	107	17.86%	Silica Dust	43	7.18%	PipeFitting	37	6.18%
			COLD COPD	82	13.69%	Formaldehyde	79	13.19%	PipeCover	50	8.35%
			Other Chronic Lung	15	2.50%	Gas Petro	222	37.06%	BuildInsulation	76	12.69%
			Pneumonia	86	14.36%	X-ray/Rad	270	45.08%	BuildDemo	47	7.85%
						Metal Process	72	12.02%	Welding	78	13.02%
						Pesticide/Herbicide	269	44.91%	Sandblasting	44	7.35%
						BisEther	65	10.85%	Fishing	9	1.50%
									Farming	104	17.36%
									LumberPaper	64	10.68%
									BoatBuild	37	6.18%
									LeatherWork	14	2.34%
									PeelCleanBoilers	36	6.01%



Cohort Statistics for Subjects who Developed Cancer N=24

Pack Year	#Subject	%Subjects	%FEV	#Subjects	%Subjects	Age	#Subjects	%Subjects	Gender	#Subjects	%Subjects	Race	#Subjects	%Subjects
<30	0	0.00%	<20	0	0.00%	45-49	1	4.17%	Female	9	37.50%	African Amer	0	0.00%
30-34	1	4.17%	20-24	0	0.00%	50-54	1	4.17%	Male	15	62.50%	African Amer Hispanic	0	0.00%
34-39	1	4.17%	25-29	1	4.17%	55-59	3	12.50%				Amer Indian, Eskimo	1	4.17%
40-44	3	12.50%	30-34	0	0.00%	60-64	5	20.83%				Eastern Indian Amer	0	0.00%
45-49	2	8.33%	35-39	2	8.33%	65-69	5	20.83%				Asian or Pacific Islander	0	0.00%
50-54	2	8.33%	40-44	1	4.17%	70-74	6	25.00%				White non-Hispanic	23	95.83%
55-59	1	4.17%	45-49	3	12.50%	75-79	2	8.33%				White Hispanic	0	0.00%
60-64	3	12.50%	50-54	2	8.33%	80-84	1	4.17%				Other	0	0.00%
65-69	1	4.17%	55-59	2	8.33%	85-89	0	0.00%						
70-74	1	4.17%	60-64	2	8.33%									
75-79	3	12.50%	65-69	11	45.83%									
80-84	0	0.00%	70-74	0	0.00%									
85-89	0	0.00%	75-79	0	0.00%									
90-94	1	4.17%	80-84	0	0.00%									
95-99	1	4.17%	85-89	0	0.00%									
100-110	2	8.33%	90-94	0	0.00%									
111-120	1	4.17%	95-99	0	0.00%									
121-130	1	4.17%												
131-140	0	0.00%												
141-150	0	0.00%												
151-160	0	0.00%												
161-170	0	0.00%												
171-180	0	0.00%												
181-190	0	0.00%												
191-200	0	0.00%												
>200	0	0.00%												

Cohort Statistics for Patients who Developed Cancer N=24

Family Cancer	# Subjects	% Subjects	Disease	# Subjects	% Subjects	Material	# Subjects	% Subjects	Industry	# Subjects	% Subjects
Family Cancers	17	70.83%	Cancer	2	8.33%	Coal	5	20.83%	UranMine	0	0.00%
			Asthma	1	4.17%	Dyes	1	4.17%	OpenPitMine	0	0.00%
			Asbestos	0	0.00%	Radon	0	0.00%	UndgrdCoalMine	1	4.17%
			Emphysema	7	29.17%	Nickel	3	12.50%	UndgrdHardRockMin	0	0.00%
			Tuberculosis	1	4.17%	Arsenic	1	4.17%	Smelting	1	4.17%
			Glaucoma	0	0.00%	Asbestos	3	12.50%	IronSteelMan	2	8.33%
			Hypertension	4	16.67%	Beryllium	1	4.17%	FoundryWork	1	4.17%
			Heart Arrythmia	5	20.83%	Chromium	1	4.17%	ChemPetro	2	8.33%
			Heart Attack	2	8.33%	Iron Ore	2	8.33%	AutoRepair	2	8.33%
			Chronic Bronchitis	4	16.67%	Silica Dust	2	8.33%	PipeFitting	1	4.17%
			COLDCOPD	3	12.50%	Formaldehyde	2	8.33%	PipeCover	1	4.17%
			Other Chronic Lung	1	4.17%	Gas Petro	9	37.50%	BuildInsulation	1	4.17%
			Pneumonia	1	4.17%	X-ray/Rad	12	50.00%	BuildDemo	0	0.00%
						Metal Process	1	4.17%	Welding	2	8.33%
						Pesticide/Herbicide	9	37.50%	Sandblasting	1	4.17%
						BisEther	3	12.50%	Fishing	0	0.00%
									Farming	5	20.83%
									LumberPaper	4	16.67%
									BoatBuild	2	8.33%
									LeatherWork	1	4.17%
									PeelCleanBoilers	0	0.00%

## **APPENDIX B**

### **The Specific Role of Isoflavones in Reducing Hormonal & Proliferative Risk Parameters in Prostate Cancer**

**Nagi Kumar, Ph.D.**

**The Specific Role of Genistein in reducing hormonal and proliferative risk parameters in Prostate Cancer.**(Kumar NB) Kumar NB, Pow-Sang J, Besterman-Dahan, K, Cantor A. (AICR. 99 BO3O (2000-2002) & DOD US Army- Advanced Cancer Detection Center Grant)

## 1.0 INTRODUCTION

The most promising approach to cancer control is a national commitment to prevention. In recent years, the role of nutrients as chemopreventive agents has received due attention. There is increasing evidence that soy phytoestrogens, also referred to as isoflavones, in addition to possessing antiproliferative properties, may alter the plasma concentration, production, metabolism and excretion of testosterone and estrogens and their impact on target tissues like prostate. Definitive prospective studies testing the effects of specific components of isoflavones that alter hormonal and proliferative biomarkers that are implicated in the promotion of prostate cancer are not available.

This is a controlled, randomized clinical trial where omnivorous men aged 50 to 80 with grade 1 & 2 prostate cancer, who meet the criteria for observation with no other treatment, will be randomized to an experimental group supplemented with isoflavones (60 mgs/day for 12 weeks) or to a control group, consuming a placebo for 12 weeks. The dose of isoflavones was selected based on data reflecting that the Oriental population, who have the lowest rate of breast and prostate cancers, consume an estimated 40-80 mg of isoflavones per day as part of their habitual diets as compared to 1 mg per day consumed by western populations.<sup>33</sup> Most of the clinical studies, including our previous studies, using soy isoflavones have used 1 – 2 mg isoflavones per kilogram body weight. At this dose, serum concentrations of isoflavones have increased and hormonal and proliferative modulations have been observed. The dosage established was thus based on the data from these studies.

If increased intake of isoflavones alters the sex hormone milieu and the bioavailability to target tissues; this should theoretically reduce or halt proliferation, as observed by changes in prostate-specific antigen. Thus manipulation of the diet by the addition of isoflavones may reduce further progression of prostate cancer in this population. Based on the results of this study, prophylactic therapies using dietary supplements such as isoflavones, with relatively few side effects, may also be used for high risk populations and replace the more controversial therapeutic, hormonal supplementation regimens that are currently used for prostate cancer risk reduction.

## 2.0 BODY

It is estimated that 198,100 new cases of prostate cancer will be diagnosed in the US in 2001. An estimated 31,500 deaths in 2001 are expected with this disease making prostate cancer the second leading cause of death in men in the US. Prostate cancer accounts for about 11% of male cancer-related deaths.<sup>1</sup>

A significant number of studies published over the past two decades have established that Western diets, typically high in fat and low in fiber, are mainly responsible for the high incidence of certain cancers, especially the hormone-dependent cancers. There is increasing evidence that components of plants and fiber-rich foods may play a role in the production, metabolism and the bioavailability of sex hormones and their impact on target tissues. The detection of lignans and isoflavonoids of plant origin in body fluids, with molecular structures similar to those of steroids have given us an indication that these substances could be critical modulators of the human hormonal system and hormonal action.<sup>2-6</sup>

It has been observed that sex hormones play an important role in increasing or decreasing the risk of prostate cancer. Patients with prostate cancer have been observed to have higher free testosterone (unbound) levels and lower levels of sex-hormone binding globulin (SHBG), estrone<sup>7</sup> and estradiol<sup>8</sup>, which may represent an additional risk factor. Androgens are essential for the function and growth of the prostate and are known to stimulate the proliferation of human prostatic cells. Administration of hormonal therapies has been shown to produce prostate cancer in rodents, while castration and estrogen therapy can reduce the risk of prostate cancer.<sup>8-10</sup> It is clear from recent studies that testosterone and estradiol are dominant contributors of androgenic and estrogenic activity. Furthermore, sex-hormone binding globulin, because it binds to and sequesters testosterone and estradiol, controls the bioavailability of these sex hormones. The high affinity of SHBG for binding to testosterone, more so than even for estradiol, also influences the circulatory levels of these sex steroids, their biodisposal to target cells as well as their mutual balance.<sup>11</sup> Therefore, an increase in SHBG can produce a greater decrease in free testosterone than in free estradiol. Although high free testosterone levels are observed in this population, low estrone and estradiol levels are seen. Synergism between androgens and estrogens may be an important factor in the etiology of prostate cancer.<sup>7</sup> This effect on the active estrogen/testosterone balance may be another potential autoregulatory mechanism for the protective effect of SHBG in prostate cancer.<sup>12</sup> Recent evidence suggests that SHBG can function as a hormone with a direct interaction with prostate cells.<sup>13</sup> Since the biological activity of testosterone is determined in part by the extent to which it is bound to plasma sex hormone binding globulin (SHBG), and given that serum estradiol may effect the estrogen/testosterone balance, an important factor in the etiology of prostate cancer, the next logical step would be to look for ways to increase this binding protein and estrogen levels to reduce the risk of prostate cancer and prevent progression of disease

Several natural anticarcinogens have now been identified in soybeans, such as: protease inhibitors, phytate, phytosterols, saponins, lignans and isoflavones.<sup>14-15</sup> However, our focus will be on the components solely unique to soybeans, the isoflavones, due to their similarity to estrogen both structurally and functionally. Among the isoflavones, diadzein and genistein are the major forms present in soybeans. After structural modifications by intestinal bacteria, isoflavones are converted to compounds, which possess weak estrogenic, and anti-estrogenic properties.<sup>16-17</sup> The chemopreventive agent, Tamoxifen, which has both estrogenic and anti-estrogenic properties is structurally related and may act in much the same way as isoflavones. These substances have been shown to not only influence hormonal metabolism but also

intracellular enzymes, protein synthesis, growth factor action, cell proliferation and angiogenesis.<sup>14-15,18</sup> Genistein has received a great deal of attention due to its interesting antiproliferative, estrogenic and anti-estrogenic effects.<sup>19-22</sup> Genistein also showed the highest concentration of all phytoestrogens present in the urine of Japanese men and women consuming their typical diet which is rich in soy products.<sup>23</sup> In a recent review of research regarding the effect of genistein on in vitro and in vivo models of cancer it was found that in 74% of the studies using animal models, the proliferation of mammary and prostatic tumors was significantly reduced with genistein.<sup>24-26</sup> In vitro, genistein also had an inhibitory effect on human tumor cell lines.<sup>24</sup> In another study of plant estrogens on estrogen sensitive cancer cells, genistein was found to compete with estradiol binding to estrogen receptors. It has also been postulated that plant lignans and isoflavonoid phytoestrogens may decrease aromatase activity, a cytochrome P450 enzyme thus decreasing conversion of androgens to estrone and estradiol, which may then play a protective role in the development of hormone related cancers.<sup>27</sup> Although isoflavones has many interesting anticarcinogenic properties, we intend to focus on its effects specific to hormone metabolism and biological activity specific to prostate carcinogenesis. Isoflavones have been shown to increase serum SHBG via increased hepatic synthesis which, as a result, decreases the bioavailability of testosterone.<sup>16, 28-30</sup> In addition, although isoflavones have been shown to have an antiestrogenic effect in a high estrogenic environment, it has been postulated by at least one researcher that they exert a proestrogenic effect in a low estrogenic environment.

Prostate Specific Antigen (PSA) and prostatic intraepithelial neoplasia (PIN) are considered to be the primary intermediate biomarkers for evaluating prostate cancer risk and potentially chemopreventive efficacy; however there are issues in their use.<sup>32</sup> Serum PSA, although well established as a biomarker of prostate cancer, it is not specific to neoplasia, and the research literature does not currently support that the level is directly related to the degree of neoplastic progression. The validation of PSA as an intermediate biomarker awaits further data, some of which may be obtained from the large NCI Prostate, Lung, Colorectal & Ovarian Cancer Screening Trial in which PSA is being monitored over several years in over 30,000 men.

Traditionally, clinicians have relied on PSA as a prognostic indicator in addition to tumor stage, grade and volume. Because the serum level of PSA is proportional to the volume of tumor present, PSA has become an integral part of disease management in this population. The growth of prostate cancer is exponential at all stages. Thus the doubling of the serum PSA will reflect the biological doubling rate of tumor. Several researchers propose statistical models such as PSA doubling time (PSAdt) as an indicator that offers a dynamic view of tumor progression- but again, only as a prognostic indicator and in some studies as a marker of treatment efficacy such as radiation therapy and post prostatectomy.<sup>31</sup> However in the observation population, identifying surrogate markers of tumor progression is a challenge, especially in chemoprevention trials of short duration where invasive markers such as biopsies are not justifiable, unless it is a standard of clinical practice. PSA continues to be our only non-invasive indicator of tumor volume.

We postulate that isoflavones can theoretically increase production of SHBG by the liver and bind to biologically active testosterone, thus lowering free testosterone levels and its bioavailability to target prostatic cells. With its proestrogenic property in the low estrogenic environment it can increase the levels of estradiol, thus producing the estrogen/androgen synergy that is essential for protection from prostate cancer. We wish to initially observe serum biomarkers of free testosterone, estradiol and sex-hormone binding globulin levels. We will observe changes in PSA from baseline to post-intervention. We will also ensure that PSA's are obtained from study candidates prior to digital rectal examination and in study candidates who are free of prostatitis or urinary tract infection.

## **SIGNIFICANCE**

If increased intake of isoflavones alters the sex hormone milieu and the bioavailability to target tissues, this should theoretically reduce or halt proliferation and thus tumor progression and volume, as observed by changes in prostate-specific antigen. Thus manipulation of the diet by the addition of isoflavones may reduce further progression of prostate cancer in this population. Based on the results of this study, prophylactic therapies using dietary supplements such as isoflavones, with relatively few side effects, may also be used for high risk populations and replace the more controversial therapeutic, hormonal supplementation regimens that are currently used for prostate cancer risk reduction.

## **PURPOSE:**

The purpose of this study is to evaluate the individual effectiveness of supplementing a group of grade 1-2 prostate cancer patients with a dietary supplement of the isoflavone, genistein (60mgs/day) in producing a change in risk parameters that are implicated in the promotion of prostate cancer, such as decrease in free testosterone, and increase in sex-hormone-binding globulin and estradiol and decrease in proliferation as indicated by decreasing total and percentage free Prostate Specific Antigens.

## **3.0 KEY RESEARCH ACCOMPLISHMENTS:**

As planned and outlined in our Statement of Work, we have accomplished the following:

### **Task 1: Recruitment and Data Collection:**

- a. We have pre-screened 120 subjects and recruited 69 eligible patients diagnosed with grade 1-2 prostate cancer who were consecutively admitted to the Prostate Program. This was the recruitment completed for the pilot phase of the trial. Thirty one (31) subjects have completed the study and an additional 20 are active in this study. Eighteen subjects dropped out of the study as they were not able to consume the soy and placebo supplementation.
- b. Upon eligibility, consent was obtained from all subjects  
Upon enrollment, the following baseline information has been obtained from all subjects admitted to the study.(Study Schema- Figure 1)

Study Schema – Table-1

Study Requirements	Baseline	During Intervention	Post-Intervention (end of 12 weeks or Off Study)
Anthropometric measurements (Weight, Height)	X	Every 4 weeks	X
Two Day Food Record (TDFR) <sup>1</sup>	X	Weekly	X
Sign informed consent	X		
Eligibility form	X		
History: (On Study form) Demographic information Personal & medical history Hormonal & reproductive history Exercise Smoking Alcohol use	X		
Non-fasting blood samples:			
Hormonal assays <sup>2</sup>	X		X
Total prostate specific antigen <sup>2</sup>	X		X
Study agent intake log		Daily	X
Clinic visit: Obtain study agent/return left over study agent Review completed TDFR and daily intake log		Every 4 weeks	X

1. Confirmation of the accuracy of eligibility information, including the 4-day diet records and using an initial screening form (Baseline only)
2. Demographic information, personal and medical history, hormonal and reproductive history, exercise, smoking and alcohol use history obtained by an RD using the Epidemiological Questionnaire (Baseline only).
3. Anthropometric measurements such as subject's height, weight, skinfold and circumference measurements (Baseline, week 6 and 12)
4. 30 mL Blood samples will be drawn into heparinized tubes in a non-fasting state at the same time of day, between 7:00 AM and 12:00 AM, for each individual to perform hormonal assays and total and percentage free prostate specific antigens.(Baseline, week 6 and 12). Hormonal assays will include free testosterone, sex-hormone binding globulin and estradiol at baseline, week 6 and at week 12. As there are no previous studies that have established the duration required to demonstrate change in hormonal levels with intake of isoflavones in males, we adopted the time taken to demonstrate hormonal changes with ingestion of isoflavones in female populations which is within one menstrual cycle. We had thus



established the evaluation point as 12 weeks or 3 months for both the female and male groups. The hormonal assays (radioimmunoassay) will be performed by Quest Laboratories. Blood draws are done by the Cancer Center phlebotomist and processed and shipped using standard procedures for shipping to Corning Nichols, who will perform the radioimmunoassays.

5. A biopsy and digital rectal exam will be performed by the GU program chief/oncologist for all patients entered in the study at baseline(routine),which will determines patient's admissibility to the study.
6. The participant will be provided with a 2-day diet record(TDFR) and instructed on reporting food intake, including weights/measures and methods of preparation of foods consumed using standard food models. (Baseline, weekly)
8. Changes may be anticipated in stool frequency or GI discomfort. A pre-validated Nutritional Symptoms Scale is used to monitor GI symptoms during intake of supplements on a weekly basis.
9. A Participant Tracking Form is used to monitor all activities and variables observed during the study period. Activities of each participant is vital for the study such as use of supplements, compliance to all monitors. This form, will in addition, serve as a checklist to monitor these variables for the Project Dietitians.
10. Quality control procedures for data collection and entry are ongoing.
11. Contact numbers were provided to patients

#### **Task 2: Abstraction of Medical Records Data:**

- c. We have continued to obtain patient disease related prognostic indicators from medical charts
- d. Data entry and quality control procedures have been initiated
- e. Follow-up interviews for data collection periods at mid-point and post completion of interventions have been completed for 31 subjects and the currently active 20 subjects is in progress.
- f. Weekly visits to the cancer center to obtain supplements and submission of monitoring instruments
- g. Shipping of completed patient's blood sample for hormonal assays and PSAs are ongoing

At the end of the study we will complete the data analysis. Pooled t-tests will, in addition be used to compare mean changes in intake of other nutrients, body composition parameters and nutritional symptoms at the end of Phase II. Pooled t-tests are justified in this case. Even if the data are only approximately normally distributed, the test is well known to be quite robust with respect to the normality assumption. These tests will be two-sided.

Multivariate repeated measures ANOVA will be performed on each variable with time as the repetition variable and treatment group as class variable. "Time effect", "group effect" and "time by group" interaction will be tested. If this last effect is significant, then "time effect" will only be reported separately for each group and "group effect" will only be reported separately for each time. The need to adjust for multiple testing is somewhat controversial. We will exercise some control over the multiple testing problem by comparing groups at each time point only if the

overall ANOVA is significant. We recognize that this analysis may have limited power due to missing data. For that reason the repeated measures ANOVA will be considered a secondary analysis and the methods described previously will be primary.

**Preliminary results of the data collected from the pilot phase of the study are as follows:**

- We have recruited 69 eligible subjects for the isoflavones/prostate cancer study and all have completed the study. The randomized clinical trial has been initiated and 31 subjects have completed the study and 20 are currently active in the study. A total of eight (18) subjects dropped out of the study, 16 of whom were unable to tolerate the taste of the product, and 2 dropped out as they experienced constipation. Due to the large number of drop-outs in the study, we are currently oversampling and recruiting additional subjects in this study.
- Demographic variables of the pilot study subjects is displayed in Table 1. The average age of the isoflavone group was 73.7 and the placebo group 70.8. No significant differences in baseline height, weight, Body Mass Index (BMI), smoking, family history of cancer was observed.
- Baseline Nutritional Intake of calories, fats, proteins, carbohydrates, fiber and cholesterol of both the experimental and placebo groups is displayed in Table 2. No significant differences between the groups was observed at baseline.
- Changes in surrogate marker of proliferation (Total PSA) is displayed in Table 4. Total PSA decreased an average of over 2 points in 69% of subjects in the isoflavone group compared to no decreases seen in subjects in the placebo group.
- Changes in hormonal markers are displayed in Table 3. Free testosterone decreased in 56% of subjects in the experimental group compared to 27% of subjects in the placebo group. We observed an increase in total estradiol levels in the group receiving isoflavones compared to a decrease in this marker observed in the placebo group. However, no significant changes were observed in SHBG levels.

#### **4.0 REPORTABLE OUTCOMES:**

1. **The Specific Role of Genistein in reducing hormonal and proliferative risk parameters in Prostate Cancer.** Kumar NB, Pow-Sang J, Besterman-Dahan, K, Cantor A, Seigne J & Allen K. Proc 10<sup>th</sup> Annual Research Conference, American Institute for Cancer Research, August 2000.
2. **The Specific Role of Genistein in reducing hormonal and proliferative risk parameters in Prostate Cancer.** Kumar NB, Pow-Sang J, Besterman-Dahan, K, Cantor A, Seigne J & Allen K. Proc. of the 4th Annual Symposium on Predictive Oncology and Therapy sponsored by the International Society for Preventive Oncology; 2000.

## 5.0 CONCLUSIONS:

As observed in the pilot phase of the study, preliminary results indicate that 69% of the subjects in the soy isoflavones group decreased total PSA by >2 points from baseline to end of the 12-week period compared to only increases observed in the placebo group. Although supplementation with the isoflavone did not produce an increase in serum levels of sex-hormone binding globulin (SHBG), we observed a decrease in free testosterone in 56% of the subjects in the experimental group compared to decrease observed in 27% of the subjects in the placebo group. Synergism between androgens and estrogens may be an important factor in the etiology of prostate cancer. This effect on the active estrogen/testosterone balance may be another potential autoregulatory mechanism for the protective effect of steroid hormones in prostate cancer. Our preliminary results indicate that we were able to decrease free testosterone levels and the total PSA and an increase in free estradiol in the group of subjects receiving soy isoflavones compared to the subjects in the placebo group, thus effecting surrogate markers of proliferation and steroid hormone concentrations, which are important factors in the etiology of prostate cancer.

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## 7.0 APPENDICES

TABLE 1. DEMOGRAPHIC CHARACTERISTICS OF SUBJECTS IN THE  
EXPERIMENTAL & PLACEBO GROUPS

VARIABLES	ISOFLAVONE SUPPLEMENTED (n=22)		PLACEBO (n=26)	
	$\bar{X}$	SE	$\bar{X}$	SE
Age, years	73.7	0.92	70.8	1.03
Baseline Weight, lbs.	189.2	6.02	188.2	4.90
Height, cm.	176.8	1.78	176.5	1.38
BMI kg/m(squared)	26.9	0.56	27.5	.64
Current or Ex- Smoker	77%		69%	
Family History of Cancer	80%		78%	
History of Benign prostatic neoplasia	50%		70%	

**The Specific Role of Genistein in Reducing Hormonal & Proliferative Markers in Prostate Cancer (N. Kumar)**

**TABLE 2. NUTRIENT CONSUMPTION AT BASELINE OF EXPERIMENTAL & PLACEBO GROUPS**

	Isoflavone Group(n=22)		Placebo Group(n=26)	
	Base Line Mean	SE	Base Line Mean	SE
<b>Energy Kcals</b>	1727	82.01	1877	146.3
<b>Protein (gm)</b>	80.4	4.4	79.8	7.02
<b>Fat (gm)</b>	56.6	4.4	68.7	7.55
<b>Cholesterol (mg)</b>	276.6	37.2	213.3	25.0
<b>Carbohydrates (gm)</b>	214.5	9.3	231.5	18.7
<b>Fiber (gm)</b>	15.1	0.9	15.4	1.93



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**TABLE 4. SERUM STEROID HORMONE CONCENTRATIONS AT BASELINE AND  
END OF STUDY**

	Isoflavone Group(n=16)				Placebo Group(n=11)			
	Baseline		Final Week		Baseline		Final Week	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
<b>Free Testosterone pg/ml</b>	71.9	7.89	61.82	5.18	65.4	5.82	57.56	2.65
<b>Total testosterone pg/ml</b>	412.7	47.7	368.94	41.9	397.2	30.4	352.3	29.7
<b>Total Estradiol pg/ml</b>	22.26	2.31	23.64	2.33	24.09	2.40	23.01	2.76
<b>SHBG nmol/L</b>	34.9	3.24	34.4	4.20	35.1	2.38	36.3	4.27

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**TABLE 4. CHANGE IN SURROGATE MARKERS OF PROLIFERATION FROM BASELINE TO END OF STUDY PERIOD**

	Isoflavone Baseline (n=16)		Isoflavone Post- Intervention		Placebo Baseline (n=11)		Placebo Post-Intervention	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
<b>Total PSA</b>	7.59	1.11	6.49	0.90	7.86	1.26	8.21	2.35

## **APPENDIX C**

### **Development of Moffitt Cancer Network as a Telemedicine and Teleconferencing Educational Tool for Health Care Providers**

**Jeffrey Krischer, Ph.D.**

## INTRODUCTION:

The Moffitt Cancer Network's (MCN) goal is to provide up-to-date oncology related information, resources, and education to oncology health care providers and researchers for the prevention and cure of cancer. Consistent with the aims of the Advanced Cancer Detection Center, the MCN provides access to educational programming, cancer control and clinical protocols, and a mechanism to exchange patient focused information leading to the improved detection and treatment of cancer. The MCN is health care provider focused and complements an array of existing public/lay information sources available elsewhere. It is built around the concept that oncology expertise is geographically centralized, multidisciplinary in nature and of limited availability. The MCN addresses these constraints by increasing availability through a World Wide Web-based design that enables wide access from many geographic locales. The objectives of this project are to:

- Collect and organize cancer information to provide educational content to physicians and other health care providers,

- Develop and implement software to encode video and audio to enable viewing over the Internet at a range of speeds (bandwidths),

- Implement a mechanism to deliver continuing education credits through on-line testing and automated submission/evaluation,

- Design and create a web page to permit easy sorting, searching and selection of educational programming,

- Design and create a web page to deliver physician referral information that includes submission of an electronic case record consisting of text and imaging data, and

- Provide access to case conferencing from remote locations using easily available audio/video to the desktop.

## BODY:

### **Task 1. Collect and organize cancer information to provide educational content to physicians and other health care providers. (Months 1-60).**

A schedule of events is determined in coordination with the Moffitt Office of Conference Planning, the USF Department of Education, the USF department of Continuing Medical Education and independent researchers wishing to present. These events include: Grand Rounds, the monthly meeting of the Cancer Control Research Interest Group (CCRIG), a number of national and local oncology conferences, as well as, more recently added, a number of JCAHO requirements for in-service education for nurses, physicians, and other hospital staff.

The MCN currently has 270 presentations in its library, increasing at a rate of 8.8 presentations per month on average. Additionally, 12 conferences sponsored by USF and Moffitt are also currently available online.

*Schedule videographer coverage of grand rounds and research conferences.*

The Network Coordinator in cooperation with Moffitt Department of Education compiles a

schedule of events. This schedule is used to determine the scheduling needs of the MCN videographer. The MCN videographer provides audio and video capture of these events.

*Coordinate notification of nursing, pharmacy and other health care providers continuing education presentations.*

The Moffitt Department of Education notifies the MCN of all continuing education presentations and obtains a release from all speakers that permits the distribution of their respective presentation by the MCN.

*Organize the videotaping of faculty scientific presentations for national oncology conferences.*

The notification and videotaping of national oncology conferences is scheduled in accordance with the system mentioned above, developed in coordination with the MCN and the Moffitt education department. A number of conferences have been added to the MCN library. These presentations are digitized and are made available on the MCN website. The presentations acquired by this activity are codified by continuing education, searchable by subject and grouped by their respective conference title.

*Coordinate with the Department of Education notification and scheduling of relevant conferences.*

The Moffitt Department of Education notifies the MCN of all relevant conferences and the MCN videographer is scheduled in accordance with the videotaping needs of each conference.

## **Task 2. Develop and implement software to encode video and audio to enable viewing over the Internet in a range of speeds (bandwidths). (months 1-60)**

*Explore the application of the Tag development software to support multiple video connections and the impact on network bandwidth.*

The MCN has developed a process of digitizing presentations using the Digital Renaissance Tag Composer. Through this process MCN is able to stream presenter's slides and audio simultaneously by using a Synchronized Multimedia Integration Language (SMIL) script file. MCN originally encoded presentation for distribution over ISDN speeds of 128k and modem speeds of 56k. The encoding process used previously created two-network streaming formats, one for ISDN speed connections at 128 kilobytes per second and a second format for current modem technology speeds of 56 kilobytes per second or less. Using the Real media server software, users linking to a presentation acquire the format (streaming speed) appropriate for their connection bandwidth. The server and the user's player handle this process automatically. Late in August 2000 MCN determined that the ISDN format was redundant, as it did not offer any significant improvement over the modem format due to the low frame rate of the presentations being developed (sometimes as low as one frame for every three minutes), and MCN has discontinued the encoding an ISDN bit rate media file and thus lowering the production time.

In June 2000, MCN migrated from Tag Composer to Windows Media in favor of a more efficient development and distribution process. Windows Media handles bandwidth more efficiently while drastically cutting the development time for each presentation. MCN is now working in a primarily tapeless environment, whereby presentations are captured digitally, directly to a

computer file. These presentations are then edited using a suite of tools provided by Microsoft and placed into the MCN library.

*Evaluate alternative connectivity models, including cable modem connections or access to cable networks as a means to enhance distribution of educational content.*

The MCN has evaluated multiple alternative connectivity models, including cable modems, ISDN, ADSL, and traditional T1 & T3 service lines. We have found that cable modems are an excellent method of distributing educational content. Cable modems provide a low cost, high bandwidth alternative for the user. This allows educational content to become more dynamic and interactive increasing the quality and effectiveness of the educational activity. MCN continues to explore and evaluate new connectivity models as they become available.

*Evaluate the Internet 2 as to its availability to sustain the necessary bandwidth for the Moffitt Cancer Network.*

Moffitt Cancer Center is currently connected to Internet 2 through the University of South Florida. Internet 2 is capable of sustaining the necessary bandwidth for the Moffitt Cancer Network. Development in this area will depend on the more general availability of the Internet 2 to MCN users.

*Resolve firewall and security issues to provide secure communication for clinical data as well as to adequately deal with subscriber/user requirements for security to permit desktop access.*

A firewall has been put in place to ensure secure communications for clinical data and to address user security issues. In August 2000, MCN moved towards streaming media as UDP packets, as opposed to only TCP packets. By doing so, caching of media streams is nearly eliminated. This required an extensive review of firewall issues. Data will only be available at pre-selected times and with pre-predetermined permission and authorization levels. We continue to evaluate new processes that will embellish firewall security.

*Uniform Resource Locator based on specific one-time virtual names.*

All prerecorded media will be encrypted in the near future and will have unique keys for specific use. Additional security methods are still being researched and firewall security is a priority.

*Expand the number of Authorized users to the Moffitt Cancer Network.*

Expansion of authorized users is critical to the digital convergence with MCN's on going research and development. We are now capable of delivering "On-demand", encrypted, and live media to desktops both user specific and publicly when appropriate. In addition, with the recent addition of continuing credit hours for nursing, we have opened a huge medical audience for MCN. MCN continues to expand its offerings in an effort to increase its user base. Use of the website has increased ten-fold over the past year. Expect growth of 800% over coming year.

**Task 3. Implement a mechanism to deliver continuing education credits through on-line testing and automated submission/evaluation. (Months 1-60).**

*Arrange for automated notification of Department of Education staff for each new presentation selected for the Moffitt Cancer Network.*

Prior to inclusion in the MCN, the Moffitt Department of Education reviews each presentation for quality of educational content.

*Establish ongoing procedures to obtain releases, objectives and CME questions to implement to permit encoding of presentations and inclusion onto the Moffitt Cancer Network.*

Presenters sign a release to rebroadcast prior to the videotaping of their presentation. The Moffitt Department of education works closely with the presenter and the MCN to establish objectives, determine appropriate CME questions and evaluate the overall quality of the educational content of the respective presentation. Upon the completion of this work, all information is passed to the MCN for inclusion into the MCN website for delivery to the user.

*Create documentation and procedures to collect appropriate demographics on individuals desiring CME and implement electronic automated notification of our Continuing Education Office to authorize and verify CMEs earned.*

Appropriate demographic information is collected from all individuals wishing to receive CME credit for physicians or nurses contact hours. Upon completion of a CME credit or contact hours, the MCN staff is electronically notified. The results of the activity are graded electronically and the information is forwarded to the USF Education Department if a CME credit or contact hour was in fact earned.

In July 2001 the MCN developed an automated procedure whereby the Continuing Education office is electronically notified immediately upon completion of credit requirements.

*Automatically link the Cancer Library to the acquisition process so that they are aware of new acquisitions and receive opportunities to extract key words for indexing, sorting and searching.* Upon the completion of the digitization of a presentation, the digitized presentation is forwarded to the Cancer Center Librarian for review. The Cancer Center Librarian extracts key words used for indexing, sorting and searching presentations on the MCN website. These keywords are added to the MCN website database for each respective presentation.

*Extend the CME process to include CEUs for nursing and pharmacy.*

The MCN currently offers CME credit for physicians as well as contact hours for nursing continuing professional education (CEU). The certifications are provided in cooperation with the USF College of Medicine and Nursing, respectively. We are continuing to explore the applicability of the content to other healthcare providers, such as pharmacists, and the requirements to offer continuing education credits. MCN plans to expand its credit offerings beyond CME and CEU in the coming year.

*Expand the educational content offerings to include mandatory requirements for risk analysis, HIV, infection control, etc.*

The MCN has expanded the educational offerings to include a number of JCAHO requirements for nurses, physicians and staff. These offerings are available internally to all personnel via the Moffitt Cancer Center Intranet.

**Task 4. Design and create a web page to permit easy sorting, searching and selection of educational programming. (Months 1-24)**

*Organize educational content along primary audience lines and develop a key word searching algorithm to subset for presentations.*

An algorithm has been developed allowing keyword searching. The keywords are assigned during the review of the presentation by the cancer center librarian. The algorithm is continually reviewed as to provide a more efficient search. The MCN website provides chronological ascending/descending, keyword search, search within results, presenter last name, first name, presentation date, and credit searches.

*Implement a database for key words according to a standard nomenclature, utilizing NLM MeSH headings, cancer site, etc.*

A keyword database has been created and is used by the MCN website for searching. The keywords are determined by the Cancer Center Librarian prior to the addition of a presentation to the MCN. The keywords are based on NLM MeSH standards.

*Expand implementation of Active Server Page (ASP) extensions to the multimedia hypertext (HTML) by adding onto the 'back-end' of the Web application i.) procedural language scripting and ii.) the ability to exchange information with a fully functioning database.*

ASP has been used throughout the site to produce dynamic, database driven web pages. ASP is used in all areas of the site to set procedural paths, increase security and generate dynamic content from the MCN databases. MCN has modified the website in the past year to produce a more dynamic, user friendly site.

*Expand and refine the JET database to incorporate user defined search phrases that are located within a variety of fields associated with the database, including a textual 'objectives' section, MeSH headings, cancer site, canned search categories, etc.*

The MCN has increased the capability of the Jet database to allow user defined search phrases. These phrases search for matches in the textual 'objectives' section, MeSH headings (keywords), cancer site, and canned search categories.

*Monitor utilization by remote site to evaluate the frequency and demand for various types of educational content to permit refinements and revisions to improve offerings.*

The MCN gathers extensive information in regards to use of the MCN website. This information includes website traffic, time spent, the number of presentations watched, for credit or not, and the frequency each presentation is watched.

**Task 5. Design and create a web page to deliver physician referral information that includes submission electronic case record consisting of text and imaging data. (Months 1-36)**

*Develop and implement a database to archive text and imaging data for retrieval by consulting Cancer Center physicians and integration with Moffitt Cancer Center clinical information systems.*



Since its media is stored as objects now, its future database will be based on usage of objects. As of July 2000, MCN began storing media and text, the former in two object formats based on Real and Microsoft Media.

*Develop a structured computerized clinical case description that provides a minimally relevant set of data that describes a clinical case for second opinion and consultation.*

Efforts to date have focused on image transfers and the capability to be DICOM compliant. Appropriate mechanisms have been developed along with interfaces to hospital PACS and Radiology Departments. Exploration is currently underway to exchange textual information and establish the computerized clinical case record.

*Acquire hardware and software to provide audio and video real time and time shifted streaming of case conferencing to remote locations for user viewing over secure communication links.*

In July 2000 MCN procured rack mounted dual processor servers and audio/video equipment for the purpose of providing both real-time streaming of media as well as simultaneous capture of that media for archive.

*Establish the necessary gateways and bridges to provide connections at a range of bandwidths to support remote connectivity.*

See, also Task 2. In cooperation with Moffitt Information Technology all necessary gateways and bridges have been put into place to provide local and remote connectivity at a range of bandwidths. MCN continues to review alternative models for growth.

*Design and implement web-based front ends to Moffitt Cancer Center clinical systems to permit secure access to patient information of patient's referred or submitted to case conferencing or second opinions.*

MCN and Moffitt have collaborated to create a total package for streaming media distribution. Internally, Moffitt is hardware ready to multicast media events and with the establishment of a new dedicated media server in August 2000; it has implemented a load-balanced high bandwidth portal for streaming media for both the Intranet and Internet. In addition, using specified unicast stations; MCN can deliver media events to other facilities that can multicast and therefore reducing the bandwidth load on MCN's media server. A test of the Unicast/Multicast processes is currently being planned.

**Task 6. Provide access to case conferencing from remote locations using easily available audio/video to the desktop. (Months 1-48)**

*Complete telegenetics experiment to assess feasibility and acceptability of this format for the exchange of clinical information.*

Beginning in May, 2000, patients presenting for genetic counseling services were approached to complete a brief questionnaire and determine interest in participation in the telemedicine study. The questionnaire was used to evaluate differences between individuals who were willing to participate in tele-counseling versus those who were not. 61 persons completed the questionnaire and were asked to participate in the study, of whom 48 provided informed consent and were randomized (79%). Accrual for the study was interrupted twice due to changes in

technical and genetic counseling personnel. We estimated that 23 participants who underwent both genetic counseling *and genetic testing* would be required for sufficient statistical power, based on cross-over study design (see below). We estimated that 40% of participants would undergo genetic testing, based on historical data; therefore 58 total participants would be required. Although more than 58 participants have enrolled in the study, only 13 (27%) have undergone genetic testing. This lower rate of genetic testing among individuals pursuing genetic counseling is thought due to gradual increase in the number of individuals at relatively lower risk for genetic mutation (although still elevated compared with the background population risk) who are being referred for genetic counseling services. Thus, we estimate that an additional 37 participants will need to be enrolled for sufficient statistical power. We feel that the low cost of the study in proportion to the importance of the data justifies continuation of the project.

Participants were randomized to receive initial genetic counseling either face-to-face (standard procedure) or via telemedicine. Participants who received their initial genetic counseling via the standard method received their genetic testing results and counseling via telemedicine, if they were eligible for and proceeded with testing, and vice versa (those who received initial genetic counseling via telemedicine had results disclosure and counseling face-to face, if they proceeded with testing). The same genetic specialists provided information via telemedicine or face-to-face. The information presented during the sessions was the same and did not differ from the standard information presented during these sessions.

Thirteen participants opted for genetic testing and a blood sample was collected. Samples were sent to Myriad Genetics Laboratories for testing. Thus far, 9 subjects have received results and post-test genetic counseling via face-to-face or telemedicine, whichever delivery is different from their initial session. The 4 other participants who have undergone genetic testing are currently scheduled for results disclosure and genetic counseling.

In order to evaluate potential differences between the experience of telecounseling versus face-to-face genetic counseling, all participants were asked to complete a questionnaire evaluating the initial (pre-test) genetic counseling experience. The questionnaire varied slightly depending on the delivery method used. Study participants who were eligible for and elected to proceed with genetic testing had results disclosure/post-test genetic counseling via the opposite delivery method used for pre-test genetic counseling (face-to-face or telecounseling) and completed a questionnaire evaluating the post-test genetic counseling experience. This questionnaire also varied slightly depending on the method of delivery. In order to further evaluate potential differences between the experience of telecounseling versus face-to-face counseling, the genetic specialist also completed an evaluation questionnaire after each session, pre-test and post-test, face-to-face or telemedicine. Here also, the version depended on the method of delivery.

Data collection instruments developed for this study were based on a version of instruments used previously in published studies of telemedicine. This study utilized the Moffitt genetic data system designed for genetic studies.

This is the first randomized study of the use of telemedicine for delivery of cancer genetic counseling (a few case reports exist). Data obtained thus far show general satisfaction with the use of telemedicine for this purpose and no difference in satisfaction compared with face-to-face

genetic counseling. We anticipate similar results during the remainder of the study. If so, data from this study will be useful toward additional investigation through a multi-center study in the community setting. This data may lead to the implementation of telemedicine technology to achieve improved access to services, reduced costs for services and improved quality of services in cancer genetic counseling.

An abstract describing the study was published in the American Journal of Human Genetics.<sup>i</sup> Preliminary data suggest no difference in satisfaction with genetic counseling delivered via telemedicine compared with delivery face-to-face. A manuscript is in preparation. Based on preliminary data, we have submitted a proposal to the NCI CCOP for a multi-center investigation of the use of telemedicine for delivery of cancer genetic counseling services in the community setting.

This study is collecting important data which supports the feasibility of utilizing telemedicine as a delivery method for cancer genetic counseling.

*Implement additional sites to expand this program and resolve billing issues within the context of existing laws and regulations regarding telehealth and teleconsultation programs.*

MCN has begun preliminary development of inter-institutional programs and plans to implement the programs on a small scale in the following year. Billing issues and regulations are of paramount concern, however, federal government continues to ease restrictions and increase acceptance of this technology.

*Establish the necessary gateways and bridges to provide connections at a range of bandwidths to support remote connectivity.*

See, also Task 2. At this time all necessary gateways and bridges are in place. As demand increases, additional gateways and bridges may be integrated into the current system.

*Develop tunneling or other secure links to resolve firewall issues regarding LAN configurations at both the Moffitt Cancer Center and remote sites.*

Moffitt is using Virtual Private Networks now.

*Acquire and install technology in conference centers where case conferencing generally occurs for selected clinics to permit retrieval and display of multiple images and clinical data submitted for this purpose by remote users.*

MCN has placed streaming/case conferencing equipment into the most widely used conference centers and continues to develop new case conferencing processes.

*Assess utilization of this technology to refine and revise formats and improve the quality and ease of remote access.*

As noted previously, MCN has made it a priority to improve the quality of its products. Moving towards the use of Microsoft products and its MPEG-4 streaming format has reduced labor and increased quality. MCN has enabled remote control of streaming servers and equipment.

## **KEY RESEARCH ACCOMPLISHMENTS:**

- The Moffitt Cancer Network is available to users and can be found at <http://network.moffitt.usf.edu>
- The MCN currently has 270 presentations in its library, increasing at a rate of 8.8 presentations per month on average. Additionally, 11 conferences sponsored by USF and Moffitt are also currently available online.
- Increase in average accrual rate from 2.3 presentations per month to 8.8 presentations per month over the past year.
- All approved Grand Rounds presentations have been taped by the Moffitt Multimedia Education Resources Center (MERC) for over two years preceding this report. The video had been captured on digital DVCAM 94 minute tapes. Recently, we have moved to a tapeless acquisition process.
- Since many of the presenters use only 35mm slide for their presentations, a process of creating final production audio/video Real media for streaming via TCP/IP has been developed. This process requires post-production labor and requires the best of the video's individual frames to be captured a second time to recreate higher quality computer images. MCN has made significant progress in this area and as of June 2000 has begun using presenter's PowerPoint files when ever possible to bypass the second image rendering process. This has reduced labor time from 3.5 days to about 5 hours, while increasing image quality noticeably. This labor savings is not realized when presenters are using 35mm film only. MCN is exploring new avenues to further automate the process while increasing quality.
- National oncology conferences have been taped and included in the MCN website database.
- Conferences have been subdivided into their respective presentations and are categorized searchable as well as searchable using the website database Access Jet engine. All conferences are pre-qualified for their ability to become online educational materials by the University of South Florida College of Medicine and, more recently, the University of South Florida College of Nursing.
- As of October 2000, we are able to issue credit for conference presentations.
- MCN is successfully using Microsoft MPEG-4 streaming. Not yet standardized, the newly introduced streaming format allows for embedded script and control processes within the media stream while decreasing bandwidth requirements and increasing quality.
- MCN has begun a process of evaluating user behavior patterns in efforts to work towards an "intelligent website".

## **REPORTABLE OUTCOMES:**

- patents and licenses applied for and/or issued;  
A notice of disclosure has been filed with the USF office of patents in anticipation of the completion of a patent application.

## **CONCLUSIONS:**

The purpose of this research is to create processes that allow medical professional to extend their abilities through the use of electronic media. MCN has evolved in pace with the change of that technology and because of its foresight and its dedication to purpose it has kept ahead of the technology. MCN has realized that streaming media processes are not yet capable of high

definition presentations at low bandwidth and has developed the best possible processes for producing usable educational media delivery using network technology. MCN's research into these processes has revealed the need for specific products and their uses. Several new programs will be developed to address these. For example, to cut down on the need for many new employees, MCN will be developing a broadcast program that will allow a single user to set start/stop times on a given event at a given location. In addition, this program must have a simple user interface that a cameraman will be familiar with, similar to a tape recorder. Further investigation into security processes must be addressed when MCN implements streaming from doctor to doctor in case reviews including new HIPAA requirements for medical privacy and confidentiality. Providing second opinion and expert information to referring physicians is an extremely important addition to MCN's research. While continuing education is a given, in the final analysis, it may be in the medical professional interaction that MCN becomes most useful.

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## **APPENDIX D**

### **African American Families with Inherited Breast or Ovarian Cancer**

**Rebecca Sutphen, M.D.**

African-American Families with Inherited Breast or Ovarian Cancer  
Principal Investigator: Rebecca Sutphen, M.D.

1. Introduction

Although the BRCA1 and BRCA2 genes are believed to account for the majority of inherited breast and ovarian cancer, little is known about the role of these genes among African-American cancer patients, since the majority of studies of BRCA1 and BRCA2 have been performed in Caucasian families. Similarly, little data are available regarding perception of risk and attitudes about genetic testing among African-Americans. This is a study of African-American women with 1) breast cancer diagnosed at a young age and/or 2) a family history of breast and/or ovarian cancer. All participants received pre- and post-test genetic counseling by a geneticist or genetic counselor. Complete sequencing of the BRCA1 and BRCA2 genes was performed and patients were given the option to receive results. The aims of the study were

- 1) to estimate the incidence of BRCA1/BRCA2 mutations in a sample of African American women compared to the incidence of these mutations in Caucasian women as predicted by various models;
- 2) to examine the histologic characteristics of tumors and risk factors associated with cancer in a sample of African American patients in light of what is known about risk factors in Caucasian patients;
- 3) to explore changes in participants' cancer knowledge and beliefs during the course of genetic counseling and testing.

2. Body

The study met all requirements of the Surgeon General's Human Subjects Research Review Board and final approval to open the study for enrollment was obtained in October, 2000. Accrual ended on September 30, 2001. Status of tasks included in the approved statement of work are as follows:

Task 1: Subject Identification

HLMCC cancer registry data was reviewed and identified 51 living African-American breast and ovarian cancer patients who were eligible for the study, based on personal history of breast or ovarian cancer at age 45 or younger, and an additional 17 women who were thought eligible for the study based on reported family history of breast/ovarian cancer.

Task 2: Subject Contact and Enrollment

Beginning in November, 2000, patients were invited to participate in the study either directly, at the time of follow-up appointment with their HLMCC physician or, for those patients without scheduled follow-ups, through a letter from their physician and the PI, followed by telephone contact from one of the African-American co-investigators. Of the 68 potential subjects identified through the HLM cancer registry: 3 patients were not contacted because their physician indicated that they were either dying or deceased. 2 letters were returned with no forwarding address. 6 patients were not eligible after review of family history. 4 patients were not available at the listed telephone number, with no forwarding number available. 28 potential recruits could not be reached despite several attempts. Of the 25 remaining potential recruits, 8 individuals (32%) enrolled in the study. 3 relatives of one participant and 1 relative of another participant were eligible for enrollment based on the finding of a mutation in their family member and also enrolled in the study.

### Task 3: Genetic Counseling and Genetic Testing

Genetic counseling was provided to all participants. All 12 elected to pursue genetic testing. Informed consent was obtained prior to blood sample collection. The samples were sent for laboratory testing.

### Task 4: Results Given to the Subjects

8 members of 4 families received results of the genetic testing. The other 4 participants did not pursue results.

### Task 5: Behavioral and Psychosocial Assessments

A baseline Survey I was completed by all the participants before the initial genetic counseling session. Survey II was sent approximately 1 week after the initial genetic counseling session. 7 were completed and returned.

### Task 6: Data Entry and Management

Data collection instruments developed for this study were based on validated instruments.<sup>1,2,3,4</sup> The study utilized the database system designed for genetic studies which is currently in use at HLMCC for other clinical genetics studies. Tasks 7-9 involve analyses and reports which are in preparation.

## 3. Key Research Accomplishments

### Aim 1

- mutations were identified in 2 of 8 families tested

### Aim 2

- BRCA2-associated tumor was ER/PR+; BRCA1-associated tumor was ER/PR-
- the two families with mutations had the strongest family history/highest risk estimates

### Aim 3

- baseline awareness of inherited cancer susceptibility was limited

## 4. Reportable Outcomes

- pilot data from this study will be used in a funding application for a more extended project through the Florida Cancer Genetics Network
- a manuscript is in preparation

## 5. Conclusions

Little is known regarding the role of the BRCA1 and BRCA2 genes in hereditary susceptibility to breast and ovarian cancer in African American families. Few studies have examined the cancer risk perceptions, knowledge and beliefs of African Americans regarding genetic testing for cancer susceptibility. Pilot data from this study adds to the limited information available regarding 1) incidence and types of mutations, 2) risk factors and tumor characteristics and 3) knowledge and beliefs among African Americans at risk for inherited cancer susceptibility. Pilot data from this study is important toward future investigation of these issues. Such investigations are essential for the development



of appropriate risk assessment models for use in African American families, and toward the development of appropriate educational and counseling approaches in this group:

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<sup>2</sup> Radloff, L.S., & Teri, L. (1986). Use of the Center for Epidemiologic Studies-Depression Scale with older adults. *Clinical Gerontologist*, 5, 119-136.

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<sup>4</sup> Steward & Ware, 1992. The Medical Outcome Study (MOS) Marital Functioning Scale.

## **APPENDIX E**

### **Molecular Fingerprint of STAT3 Regulated Genes for Early Detection of Human Cancer**

**Richard Jove, Ph.D.  
Emmanuel Lazaridis, Ph.D.**

## Progress Report of ACDC Project:

### Molecular Fingerprint of STAT3 Regulated Genes for Early Detection of Human

#### Cancer

PI: Richard Jove, Ph.D.

Co-PI: Emmanuel Lazaridis, Ph.D.

#### 1. Introduction

Recent advances in microarray technologies promise more specific molecular classification of tumors based on gene expression profiles, which will allow a more well-defined basis for earlier detection and selection of therapeutic strategies. In order to take advantage of this powerful new technology, characteristic gene expression profiles or "molecular fingerprints" must be identified in tumors and associated with specific disease states as well as responses to particular therapies. Our earlier studies suggested that activation of STAT3-mediated gene regulation in human tumors not only contributes to malignant progression, but also may confer resistance to chemotherapy. In the original ACDC/DOD application, we proposed that a STAT3 molecular fingerprint may be detectable at the initial stages of cellular transformation, and thus would provide a novel molecular marker for early detection of human tumors that harbor activated STAT3. In addition to detecting the presence of tumors, the STAT3 molecular fingerprint may identify the subset of tumors with more potential for malignancy and/or resistance to therapy. To determine the STAT3 molecular profile associated with oncogenesis, we are using a combination of cutting-edge microarray technology and advanced bioinformatics methodology in highly collaborative studies. The ultimate goal of the present studies is to define a set of STAT3-regulated genes associated with oncogenesis that will comprise an "OncoStatChip" suitable for clinical microarray analysis for early detection of tumors in the future.

#### 2. Body

Our ongoing work to define a subset of STAT3-regulated genes comprising the STAT3 molecular signature has nearly reached its conclusion. Laboratory experiments have been completed and data from all conditions are being collected and processed to define a final STAT3 fingerprint. As we did not obtain formal approval from DOD for the patient specimen studies, we focused our experiments on the cell line models. The patient specimens studies will be completed separately as part of an NCI-funded, IRB-approved P01 program project grant (Molecular Oncology Program Project), and then combined with the results of this DOD-funded study for final publication. For the present DOD-funded experiments, we have characterized various oncogenically transformed cell lines of rat, mouse, and human origin that display various levels of STAT3 activation. Our experimental design has utilized numerous independent methods of modulating the levels of STAT3 activity in multiple cell lines. Using this approach we will increase the

probability of identifying STAT3-regulated genes, while at the same time eliminating those genes that may be species or cell-type specific. This multi-step approach (Figure 1) initially identifies a large set of potential STAT3 regulated genes in cell lines established from multiple species. This list is then refined by examination of human breast epithelial carcinoma cell lines. The final STAT3 list is compiled by modulating the level of STAT3 activity in these cell lines using tyrosine kinase inhibitors, growth factors, antisense STAT3 oligonucleotides, and dominant-negative STAT3 (STAT3 $\square$ ) protein.

## Task 1.

To begin defining the list of potential STAT3 regulated genes, three independent murine fibroblast cell lines were examined, NIH3T3, Balb/c3T3, and 3Y1, using Affymetrix GeneChip microarray technology. Two conditions from each cell line were analyzed, normal exponentially-growing cells and cells that were stably transformed with the viral oncoprotein, v-Src, a potent activator of STAT3 (Yu et al., 1995). Total RNA for all samples was harvested using the RNeasy procedure (Qiagen) that yields high-quality RNA. Purified RNA samples were then prepared for microarray analysis using the recommended Affymetrix protocols. Labeled rat samples (3Y1) were hybridized to a RGU34A chip composed of approximately 7,000 genes and mouse samples (NIH3T3 and Balb/c3T3) were hybridized to MU11K (A and B) chips, each composed of 6,500 genes. The data generated by the Affymetrix software was then saved as "average difference values" in a tab-delimited text file for further analysis. Data from normal and v-Src transformed cells was then imported into GeneSpring, a microarray data analysis software package (Silicon Genetics).

Expression values from v-Src transformed 3Y1 fibroblasts were then compared to its normal counterpart and genes that displayed altered expression greater than 2-fold increase or decrease were identified for further analysis. From a list of 7,000 candidates, 1,481 rat genes were identified that displayed altered expression meeting the 2-fold restriction.

Data from normal and v-Src transformed mouse fibroblasts were similarly analyzed using the GeneSpring software. Figure 2 shows that 135 genes displayed at least a 2-fold increase in both NIH3T3 and Balb/c3T3 cell lines. In addition, 168 genes displayed a 2-fold or greater reduced expression as a result of v-Src transformation in both cell lines. Many genes contained within these lists, such as TGF, nucleolin, transin, DRS, ornithine decarboxylase, fibronectin and collagen have been previously shown to be regulated by the Src oncoprotein (Birchenaill, 1990; Gillet, 1990; Matrisian 1985; Kahana, 1985; Tyagi, 1985; Pan, 1996). The presence of these marker genes increases our confidence in the data obtained from the Affymetrix technology. Genes that have been previously described as STAT3 targets, for example Cyclin D1, WAF1, SOCS-3 and VEGF, also appeared on our filtered lists, providing further confirmation of the GeneChip technology and our data analysis methods (Naka et al., 1997; Sinibaldi et al., 2000; Funamoto et al., 2000). There are also many EST's and previously undocumented genes that represent potentially novel Stat3-regulated genes. There was also some overlap between the rat and mouse cell lines (Figure 3); however, this comparison is substantially more

complicated because accession numbers are unique for genes with the same names from different species.

Data was median-centered and hierarchical clustering was performed using the Cluster software package from the Eisen Lab at Stanford University. Figure 4 illustrates that the v-Src transformed cell lines cluster together independent of their normal counterparts. This further reinforces the fact that similar gene expression patterns are present in both v-Src transformed cell lines with activated STAT3. Examination of gene clusters reveals the existence of two major clusters that appear to be regulated by v-Src. One cluster represents genes that display increased expression in response to v-Src transformation, while the other cluster represents genes that decrease in expression. To further refine our list, v-Src transformed NIH3T3 and Balb/c3T3 cells were also transfected with antisense STAT3 oligonucleotides and dominant-negative STAT3 (STAT3 $\Delta$ ) protein to block STAT3-mediated gene expression. Cluster analysis and 2-fold filtering are still in progress for these samples.

Creating a list of v-Src regulated genes in mouse fibroblasts is intended to provide a foundation for defining a STAT3 "molecular fingerprint". The genes that comprise this list serve three major purposes. First, it reduces the number of genes of interest to a more feasible number. Second, it allows us an opportunity to partially validate the microarray technology due to the fact that many gene targets of v-Src transformation have already been well characterized. Finally, it allows us to identify genes that are regulated by STAT3 across different species. Matching orthologous genes from mouse to human can be a challenging task. For genes that do not share a common name or function we utilize a sequence Blast search to determine the closest match. This technique allows us to match many genes that would have otherwise been missed.

Our studies investigating human STAT3-mediated gene regulation have focused on breast epithelial carcinoma cell lines. We have characterized 4 human cell lines, 2 that display elevated STAT3 activity (MDA-MB-231, MDA-MB-468), and 2 that do not (MDA-MB-361, MDA-MB-453) (Garcia et al., 2001). Total RNA was harvested from each of the cell lines and hybridized to U95A Affymetrix chips representing 13,000 human genes. The data were imported into GeneSpring software and the genes that displayed altered expression between STAT3-negative and STAT3-positive cell lines were identified. 359 genes showed at least a 2-fold increased expression in all cell lines and 272 genes showed 2-fold decreased expression (Figure 5). The data were then analyzed using Eisen's hierarchical clustering and experimental tree nodes indicate that the two STAT3-negative cell lines cluster independently of the STAT3-positive cell lines (Figure 6). This suggests that the breast epithelial cell lines examined show differential gene expression based on STAT3 activity status. The genes that display altered regulation correlating with STAT3 activity status were then selected as likely candidates of STAT3 regulation. To further increase the confidence that the genes identified are regulated by STAT3, we have utilized multiple modulators of STAT3 status. To inhibit STAT3 signaling, cell lines were treated with two pharmacologic inhibitors of STAT3 signaling, the tyrosine kinase inhibitors AG490 and PD180970, which we previously showed block STAT3 activity in human breast carcinoma cell lines (Garcia et al., 2001).

In addition, antisense STAT3 oligonucleotides and STAT3 $\square$  the dominant-negative form of STAT3, were also used. Moreover, STAT3 signaling was further induced with the growth factor EGF and blocked with the EGF receptor inhibitor PD158780 (Garcia et al., 2001). All of the experiments involving the use of modulators of STAT3 activity have been completed and the data are still being processed for the cell lines examined in this study.

One cell line in particular, MDA-MB-468, has the most complete set of processed data sets and was analyzed using GeneSpring and Eisen's Cluster software. Comparison between the MDA-MB-468 cells treated with PD180970 and STAT3 $\square$  show that 150 and 166 genes display altered expression of at least 2-fold increase or decrease, respectively (Figure 7). When the EGF and PD158780 treated cells are included in the comparison, the results define a relatively small list of genes that correspond to genes altered by PD180970 and STAT3 $\square$ . Cluster analysis of the six independent conditions confirms these findings (Figure 8). The PD180970 and STAT3 $\square$  cluster on the same node, suggesting that these two treatments affect many of the same genes. However, the EGF treated cells cluster independently, suggesting that this growth factor elicits a very different gene expression pattern than the other treatments. As data from the remainder of the treatment conditions are processed, we will establish a more complete list of the genes that are most consistently regulated by STAT3.

## Task 2.

Statistical analysis of our data is currently in the process of being completed. The Affymetrix technology is considerably more sophisticated than data generated by cDNA spotted arrays. Each gene represented in the Affymetrix microarray corresponds to a calculated value from 20 unique probe sets. Based on this complexity, we have had to develop novel approaches to re-evaluate the procedure for interpreting data. We have designed software that allows extraction of raw data generated from each of the unique probe sets for each gene. Access to raw data has enabled us to compare various methods of gene expression calculations. We developed a novel method for calculating gene expression summaries from probe sets as part of this work (Lazaridis et al., 2001). We employ a non-parametric approach to weighing oligo features using a minimum risk criterion, an approach that is easily described and implemented. Without loss of generality, consider a single probe set. One can think of oligos in this probe set as players on a team selected on evidence that as individuals they are top performers. The performance of any given player can be gauged according to how well that player estimates a value of interest, in this case, the expression of the gene associated with the probe set. Each microarray instance in a particular analysis corresponds to a single game, resulting in a score for each of the members of the team. Our main question involves a coaching decision, whereby the analyst (coach) seeks to obtain a better estimate of (unobserved) gene expression in each sample,  $\theta_j$ , for a particular probe set.

Formally, let  $i$  index  $N$  players and  $j$  index  $J$  games. Denote the response (average intensity) of each player (oligo) at each game (microarray instance) by  $y_{ij}$ . It can easily

be shown that a coach should use  $\bar{y}_{\bullet j}$  to estimate  $\theta_j$  in the situation where all the players perform equally well across games. Let  $\phi_i y_{ij}$  be the coach's estimate of  $\theta_j$ ,  $\phi_i$  being a parameter that corresponds to the coach's evaluation of player  $i$ . Values of  $\phi_i$  near 1 suggest that the coach believes player  $i$  to be a good unbiased estimator of the  $\theta_j$  over the  $J$  games. Values larger than 1 suggest that the coach believes player  $i$  to be a low biased estimator of  $\theta_j$ . Values near 0 suggest that the coach believes player  $i$  either estimates  $\theta_j$  too high, or perhaps, that player  $i$  is so inconsistent over games as to require this player's contribution be highly discounted.

In the microarray context, relative performance of players is unknown a priori and must be estimated by the coach. Since the values of  $\theta_j$  are unknown, exact loss functions cannot be calculated. Instead, it is reasonable to suppose that the rational coach should evaluate each player against a best estimate of gene expression derived from the rest of the team.

Assuming equal importance of games played, the problem of minimizing loss over players and games can be addressed by calculating a set of parameters,  $\phi_i$ , such that a sum of squares loss over players and games is minimized.

$$S(\phi; y) = \sum_i \sum_j \left( \phi_i y_{ij} - \frac{\sum_{k: k \neq i} \phi_k y_{kj}}{\phi_{\bullet} - \phi_i} \right)^2$$

This procedure may be applied to each probe set on one or more microarray instances. The  $\phi_i$  parameters are not normalized in our procedure.

Minimization can be performed using conjugate gradient methods available in many packages. After prototyping in MathCAD, we employed the `nlminb()` function in S-Plus to minimize this expression in terms of  $\phi$ . Because of speed issues in S-Plus, we are presently testing a C routine to minimize this function. We have also tested `proc nlp` in the SAS System with excellent results.

We are currently evaluating this and other algorithms to determine the best combination of methods to represent true gene expression. All microarray technology and data analytic methods have inherent limitations, requiring that inferences regarding gene expression profiles be confirmed by an independent method. Although Northern blot analysis could be adequate, the most quantitative and accurate measure of mRNA expression is real-time PCR. In our studies, we are verifying key genes identified in the STAT molecular signatures by real-time PCR experiments. This technology has provided us with a gold standard for comparison with the Affymetrix GeneChip data.

Following completion of data processing, latent class analysis will be utilized as described in the original proposal to provide identification and classification of genes that may have been missed using other analytical methods. These design elements will be extremely valuable in refining our list of genes that are STAT3 regulated in breast cancer.

### Task 3.

Although we intended to build an interface to latent class analysis that would be amenable to use by biologists as part of this proposal, we discovered that our intention was premature because of the technical issues that needed to be addressed in the context of analyzing Affymetrix data. The two major hurdles were in the estimation of gene expression, which we addressed above, and in our capacity to perform analyses on many thousands of microarray features.

We have been addressing the latter problem by building a web-based system over an Oracle backend for managing and processing Affymetrix data. Eventually, it is envisioned that this system will integrate with statistical tools for further downstream analyses, including latent class analysis. At the present time, the system is used to manage and process data from this and other microarray studies across the Moffitt.

We present some screen-shots of this system in the Appendix. Figure 9 illustrates that the system manages information from across the many chip designs available through Affymetrix, 5 of which were used during this study. The system is able to process many data files at the same time, as illustrated by the many java processes shown to be running on the system in the next shot. Investigators can access the system from anywhere in the world at any time of the day or night to work with their data. Figure 10 shows the part of the interface for submitting a new set of data to the system. Processing of each human U95A chip takes approximately 4 hours on this system, which informs the user by e-mail when the process is complete. Finally in Figure 11, we show the user interface for conducting basic queries of the system, which may be used to interactively investigate the data. Dr. Lazaridis is continuing the process of building the capabilities of this system.

### 3. Key Research Accomplishments

- ◆ Identified 303 unique mouse genes which display altered regulation by v-Src in the context of STAT3 activation and oncogenesis.
- ◆ Hierarchical clustering illustrates that gene expression patterns of v-Src transformed cell lines with activated STAT3 cluster together regardless of cell type.
- ◆ Identified 631 human genes that display altered expression associated with STAT3 activation in breast cancer.
- ◆ Implemented and tested software that extracts raw data from Affymetrix files.
- ◆ Developed and published a novel approach for obtaining gene expression estimates from raw probe set data.
- ◆ Constructed an enterprise-capable interface and data management system for Affymetrix data.



#### 4. Reportable Outcomes

An abstract was submitted to the Oncogenomics meeting held in Tucson, Arizona, in January of 2001. This meeting was sponsored by the American Association for Cancer Research and Nature Genetics, and is one of the major international meetings on the application of microarray gene expression profiling to cancer. Our abstract was one of the few selected for a platform talk. An abstract was also submitted to the Oncogene meeting held in Fredrick, Maryland in June 2001, and selected for a poster presentation. Both abstracts, which are attached as appendixes, were presented by Dr. Dominic Sinibaldi, who is the Postdoctoral Fellow bridging the research groups of Dr. Jove and Dr. Lazaridis on this project by performing laboratory experiments and participating in the data analysis.

The manuscript, "A Simple Method to Improve Probe Set Estimates from Oligonucleotide Arrays", has been accepted for publication in *Mathematical Biosciences*.

An NIH R01 grant application is currently in preparation to extend the studies funded by this ACDC/DOD project. The application will have Dr. Jove as the Principal Investigator and Dr. Lazaridis as a Co-Investigator and will be submitted for November 1, 2001. We anticipate that the data analysis to define the final STAT3 molecular signature will be completed within 3 months and that a manuscript describing this work will be submitted for publication in January, 2002. Dr. Dominic Sinibaldi has received outstanding training in cutting-edge molecular biology and bioinformatics while working as a Postdoctoral Fellow on this project, which has prepared him well for assuming a leadership position in the field as an independent investigator.

#### 5. Conclusions

We have analyzed various cell lines from multiple species for altered gene regulation associated with STAT3 activation in oncogenesis using Affymetrix microarray technology. Data have been generated from 39 different microarray chips. Comparison of these data by a variety of analytic methods has resulted in the identification of a subset of genes that are regulated by STAT3 in the context of v-Src oncogenesis and human breast cancer. The final analysis of cell lines with manipulated STAT3 signaling status is in progress to further refine our list of genes, which will ultimately comprise a STAT3 molecular fingerprint for breast cancer. Comparisons among all of the STAT3-associated gene expression profiles will define the subset of genes comprising the "OncoStatChip" that will be evaluated as molecular markers for early detection of breast cancer. Ultimately, we anticipate that the STAT3 molecular fingerprint will provide valuable information for improved diagnostics and treatment of breast cancer.

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## 7. Appendices:

- A. Oncogenomics Conference Abstract, presented at Tucson, AZ, in January, 2001
- B. Oncogene Conference Abstract, presented at Frederick, MD, in June, 2001.
- C. A Simple Method to Improve Probe Set Estimates from Oligonucleotide Arrays, *Mathematical Biosciences* (In Press)



Figure 1. Multi-step process in defining a STAT3 molecular fingerprint. Microarray analysis is used to first identify a large number of potential STAT3 regulated genes using murine cell lines. The list is refined by shifting focus to human cell lines and by modulating the STAT3 activity. Real-time PCR is utilized to confirm data obtained from microarray experiments.

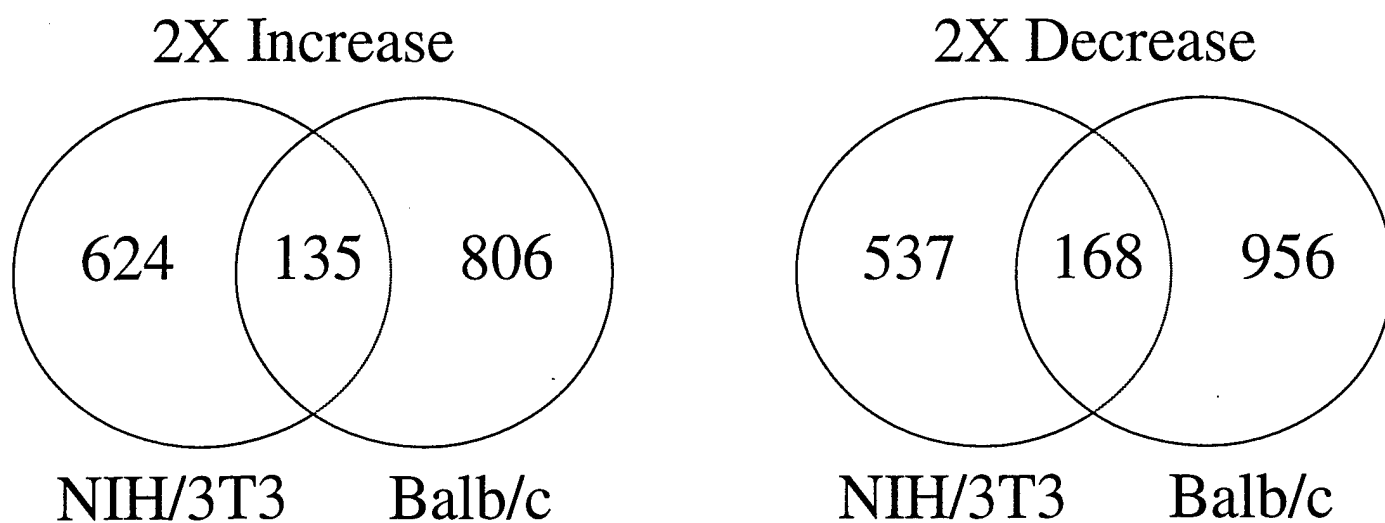


Figure 2. Venn diagram of v-Src regulated genes in mouse fibroblasts using GeneSpring. v-Src transformed cell lines were compared to their normal counterparts and genes that displayed 2-fold increased or decreased expression changes were selected. Genes that were found to be present in NIH/3T3 and Balb/c3T3 cells were then compared and the genes in common were identified.

<i>Gene</i>	<i>NIH 3T3</i>	<i>Balb /c3T3</i>	<i>3Y1</i>
<b>N-myc</b>	125	31	
<b>VEGF</b>	11	6	19.1
<b>SOCS-3</b>	9	11	19
<b>Fra-1</b>	4	218	4
<b>metallothionein</b>	2	4	5
<b>tenascin-X</b>	4	12	31
<b>transferrin recep</b>	5	4	16
<b>Cyclin D1</b>	4	5	
<b>osteopontin</b>	3	4	
<b>tob family</b>	3	3	
<b>plakoglobin</b>	-3	-23	
<b>inhibin <math>\beta</math>-A</b>	-4	-3	
<b>selenoprotein P</b>	-14	-21	
<b>Hes-1</b>	-21	-3	-28
<b>PDGF-<math>\alpha</math></b>	-4	-20	

Figure 3. Table of genes that have been previously linked to v-Src transformation, STAT3 regulation or oncogenesis. Values are expressed as fold changes in expression between v-Src transformed and normal counterpart cell lines.

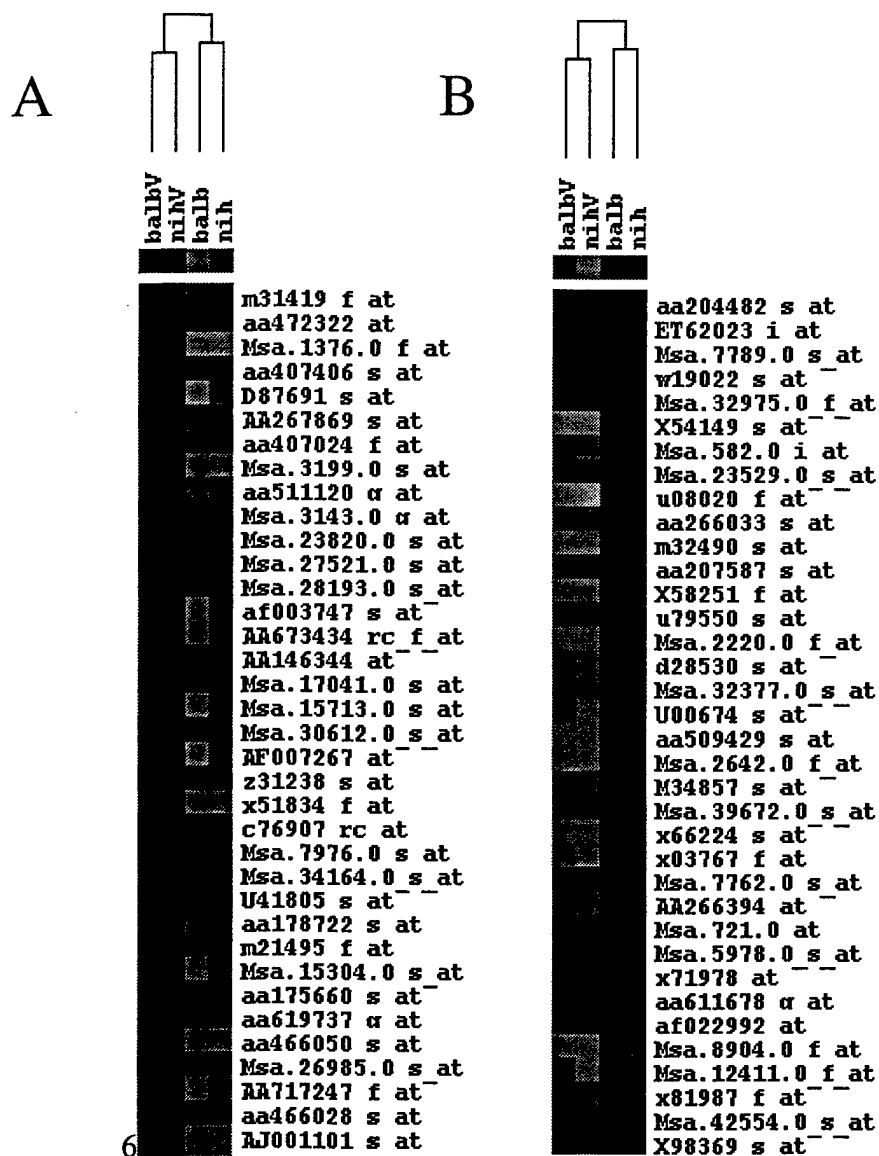


Figure 4. Hierarchical clustering of v-Src transformed mouse cell lines. Affymetrix data was imported into Cluster (Eisen) and data was filtered to include those genes which displayed absolute value changes in expression  $\geq 3$ -fold over background. The data were then median centered and processed using complete linkage clustering. Two partial clusters are displayed: (A) depicting genes that increase in expression as a result of v-Src transformation, and (B) genes that decrease.

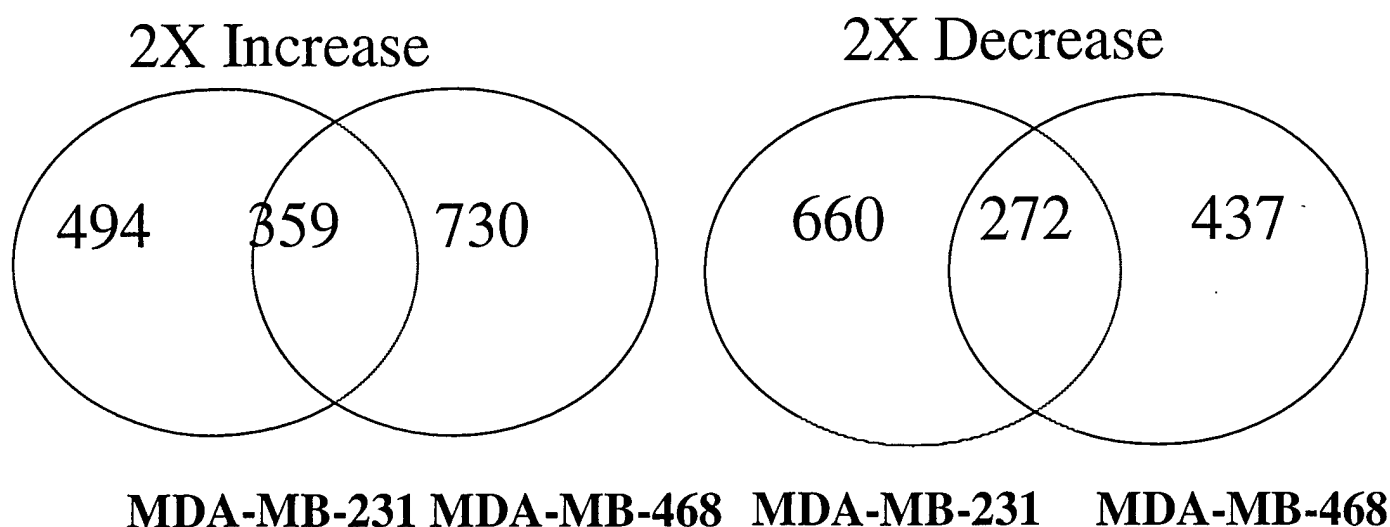


Figure 5. Venn diagram of potential STAT3 regulated genes in human epithelial carcinoma cell lines using GeneSpring. Stat3-positive MDA-MB-231 and MDA-MB-468 cells were compared to two STAT3-negative cell lines (MDA-MB-453, MDA-MB-361) and genes that displayed 2-fold increased or decreased expression changes were selected. Genes that met this requirement for each cell line were then compared and the genes in common were identified.

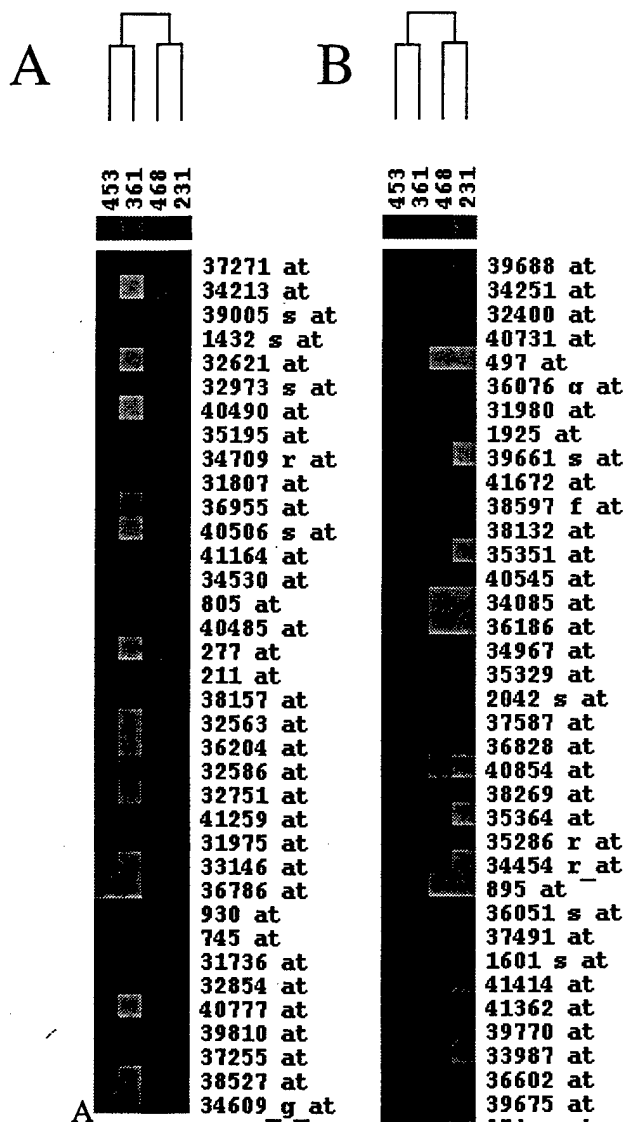


Figure 6. Hierarchical clustering of breast epithelial carcinoma cell lines. Affymetrix data was imported into Cluster (Eisen) and data was filtered to only include those genes which displayed absolute value changes in expression  $\geq 3$ -fold over background. The data were then median-centered and processed using complete linkage clustering. Two partial clusters are displayed: (A) depicting genes that increase in expression corresponding to activated STAT3 signaling, and (B) genes that decrease.



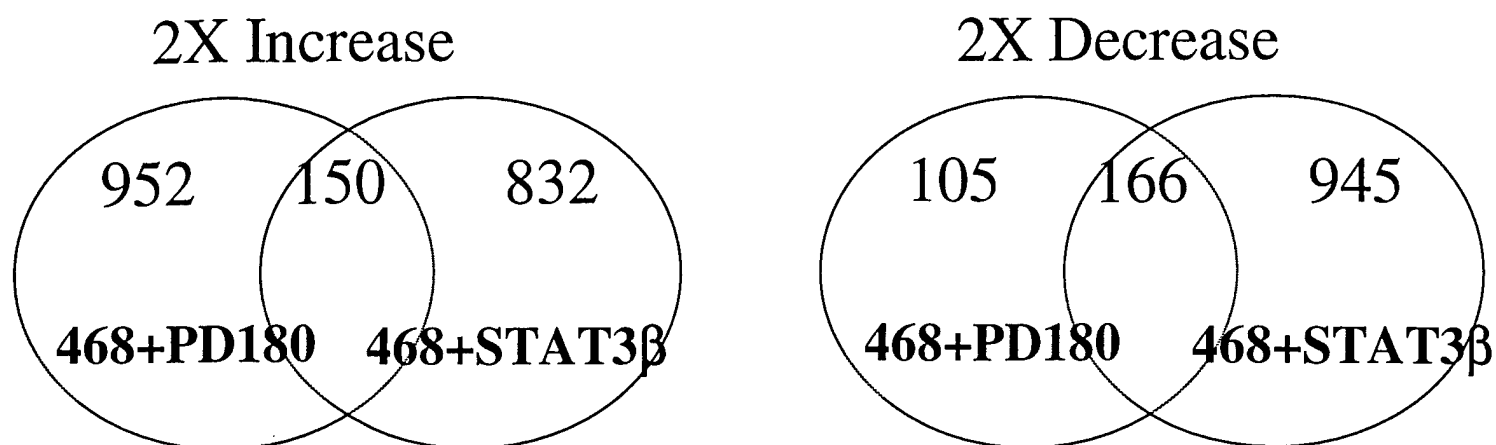


Figure 7. Venn diagram of genes that display altered expression by inhibition of STAT3 signaling. MDA-MB-468 cells were compared to same cells treated with PD180970 for 72 hours, and genes that displayed 2-fold increased or decreased expression changes were selected. MDA-MB-468 infected with a STAT3 $\square$  adenovirus were compared to cells infected with empty vector virus only. Genes that that met the 2-fold restriction for each treatment condition were then compared and the genes in common were identified.

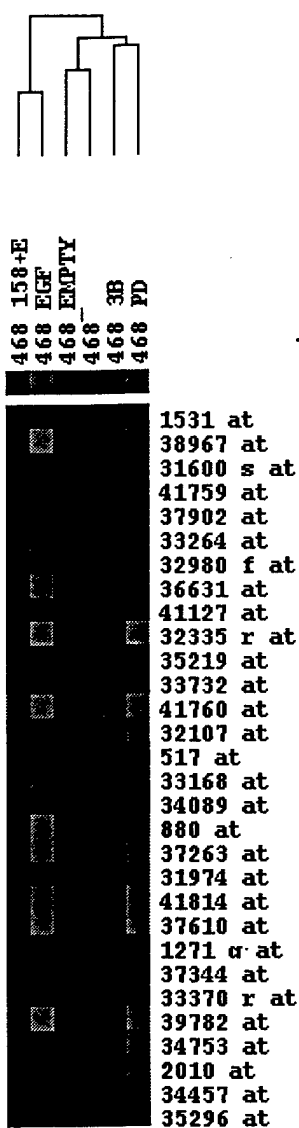



Figure 8. Hierarchical clustering of MDA-MB-468 cells treated with inhibitors of STAT3 signaling. Affymetrix data was imported into Cluster (Eisen) and data was filtered to only include those genes which displayed absolute value changes in expression  $\geq 3$ -fold over background. The data was then median-centered and processed using complete linkage clustering. Cells were treated with PD158780 and EGF (158+E), EGF, empty virus (Empty), untreated (468), STAT3 $\square$  virus (3B) or PD180970.



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CDF File	Description	Version	Chip Name	Rows	Columns	Entries
HG_U95A.CDF	U95A Chip	GC3.0	HG_U95A	640	640	407448
Hu6800.CDF	Hu6800 Chip	GC2.0	3101_a03	536	536	284915
HG_U95B.CDF	U95B Chip	GC3.0	HG_U95B	640	640	407558
HG_U95C.CDF	U95C Chip	GC3.0	HG_U95C	640	640	407568
HG_U95D.CDF	U95D Chip	GC3.0	HG_U95D	640	640	407550
HG_U95E.CDF	U95E Chip	GC3.0	HG_U95E	640	640	407560
Mu11KsubA.CDF	Mouse 11K Subunit A Chip	GC3.0	Mu11ka_a	534	534	92100
Mu11KsubB.CDF	Mouse 11K Subunit B Chip	GC3.0	Mu11kb_a	534	534	92100
Mu19KsubA.CDF	Mouse 19K Subunit A Chip	GC3.0	Mu19ka_a	534	534	92100
Mu6500subA.CDF	Mouse 6500 Subunit A Chip	GC2.0	1252aa01	260	260	66637
Mu6500subB.CDF	Mouse 6500 Subunit B Chip	GC2.0	1252ba01	260	260	66669
Mu6500subD.CDF	Mouse 6500 Subunit D Chip	GC2.0	1252da01	260	260	66633
Mu6500subC.CDF	Mouse 6500 Subunit C Chip	GC2.0	1252ca01	260	260	66657
MG_U74A.CDF	Mouse U74 Chip	GC3.0	MG_U74A	640	640	88100
RN_U34.CDF	Rat N U34 Chip	GC3.0	m1811	218	218	44916
Mu19KsubB.CDF	Mouse 19K Subunit B Chip	GC3.0	Mu19kb_a	534	534	88100
Mu19KsubC.CDF	Mouse 19K Subunit C Chip	GC3.0	Mu19kc_a	534	534	88100
RT_U34.CDF	Rat T U34 Chip	GC3.0	1807_a75	218	218	43200
RG_U34A.CDF	Rat G U34A Chip	GC3.0	10163a75	534	534	84100

Submit Reset

Total number of rows in the CDF data table: 3302011  
Total number of rows expected: 3302011

sun-midas1.moffitt.usf.edu - default - SSH Secure Shell

File Edit View Window Help

Quick Connect Profiles

last pid: 24373; load averages: 1.60, 1.73, 1.35 09:31:57

58 processes: 51 sleeping, 6 running, 1 on cpu

CPU: 46.8% idle, 43.2% user, 10.0% kernel, 0.0% iowait, 0.0% swap

Memory: 512M real, 106M free, 306M swap in use, 2112M swap free

PID	USERNAME	THR	PRI	NICE	SIZE	RES	STATE	TIME	CPU	COMMAND
24295	nobody	8	48	0	64M	35M	run	0:46	6.62%	java
24213	nobody	8	57	0	64M	35M	run	1:21	5.47%	java
24245	nobody	8	48	0	64M	35M	run	1:19	5.32%	java
24356	nobody	8	46	0	64M	35M	run	0:39	5.21%	java
24229	nobody	8	0	0	64M	35M	sleep	1:21	5.20%	java
24193	nobody	8	0	0	64M	35M	sleep	2:06	5.14%	java
24263	nobody	8	48	0	64M	35M	run	1:15	5.03%	java
24339	nobody	8	48	0	64M	35M	run	0:39	5.00%	java
24323	nobody	8	20	0	64M	35M	sleep	0:38	4.72%	java
24373	leazarlen	1	58	0	2544K	1616K	cpu	0:00	0.19%	top
24144	root	1	58	0	2960K	1952K	sleep	0:00	0.01%	sshd2
18113	root	1	24	0	2960K	1184K	sleep	2:57	0.00%	sshd2
23860	root	51	0	0	84M	53M	sleep	2:13	0.00%	java
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18769	root	1	18	0	2960K	1184K	sleep	0:35	0.00%	sshd2

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Figure 9. Screen image showing ability of MIDAS to process multiple types of Affymetrix chips.

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Client File

File Description

Store in Project

Project Key	Description	Authorization String	Subproject Key
<input checked="" type="radio"/>	Metastasis PooledTissueStudy	Affy data in this area pertains to the Yeatman Metastasis study employing mixtures of various tissues.	201

Associate with Chip Definition File

CDF File	Description	Version	Chip Name	Rows	Columns	Entries
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<input checked="" type="radio"/> HG_U95B.CDF	U95B Chip	GC3.0	HG_U95B	640	640	407558
<input checked="" type="radio"/> HG_U95C.CDF	U95C Chip	GC3.0	HG_U95C	640	640	407568
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<input checked="" type="radio"/> Mu11KsubA.CDF	Mouse 11K Subunit A Chip	GC3.0	Mu11ka_a	534	534	92100
<input checked="" type="radio"/> Mu11KsubB.CDF	Mouse 11K Subunit B Chip	GC3.0	Mu11kb_a	534	534	92100

**CDF File Upload Completed - Message (Plain Text)**

File Edit View Insert Format Tools Actions Help

Reply Reply to All Forward

From: MIDAS Web [mailto:midas@usf.edu] Sent: Friday, June 15, 2001 1:05 PM  
To: Emmanuel  
Subject: CDF File Upload Completed

Dear Emmanuel:

Recently you requested batch processing of an Affymetrix Chip Definition File (CDF). The unique identifier established for this CDF is 2746488. Please include this key in all correspondence concerning this transaction.

The file was successfully loaded and processed: 43200 rows were stored in the database.

MIDAS Web

Figure 10. MIDAS screen image showing interface for project organization and up-loading of cel files for storage.

MIDAS Center at USF - Microsoft Internet Explorer


File Edit View Favorites Tools Help

Back Forward Stop Search Favorites History

Links Customize Links Free Hotmail Windows

Address http://www.midas.usf.edu/ Go

Y Messenger My Yahoo! Yahoo! Finance Yahoo! Mail News Shopping Entertainment Travel



# MIDAS

Interdisciplinary Center at the University of South Florida

Mathematical modeling of Image Data Across the Sciences

Back Home

- General
- Helpful Resources
- Authorized Tools
  - Register
  - Login
  - Manage Affy CDE
  - Manage Affy Data
- Manage Users
- Logout
- Administrative Tools
- Links

## QUERY A CHIP DEFINITION FOR A SPECIFIC GENE

### TOOLBOX: AFFYMETRIX

Please specify information concerning the gene of interest. Note that precedence to fields is given top to bottom. Thus, if a gene with the given Affymetrix identifier is found, the description substring is ignored by the query.

Gene #1

Affymetrix Identifier  ☐ = ☐ like

or Description

Gene #2

Affymetrix Identifier  ☐ = ☐ like

or Description

Gene #3

Affymetrix Identifier  ☐ = ☐ like

or Description

Gene #4

Affymetrix Identifier  ☐ = ☐ like

or Description

Internet

Figure 11. Screen image illustrating ability of MIDAS to query processed cell files based on gene identifier or description.

A. (Oncogenomics Meeting Abstract) Defining a Molecular Fingerprint of STAT3 Regulated Genes Associated with Oncogenesis using Microarray Technology and Novel Statistical Methods

Dominic Sinibaldi, Roy Garcia, Greg Bloom, Peter Geiser, Susan Minton, Carlos Muro-Cacho, Emmanuel Lazaridis, and Richard Jove. H. Lee Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine, Tampa, FL 33612.

Signal Transducers and Activators of Transcription (STATs) are latent cytoplasmic transcription factors that are involved in normal cytokine and growth factor signaling. Recent studies have shown that certain STAT family members, most notably STAT3, are constitutively activated by various oncoproteins such as Src. Furthermore, STAT3 signaling is constitutively activated with high frequency in diverse human tumors including breast carcinoma, head and neck squamous cell carcinoma, multiple myeloma, and various leukemias. These findings indicate that downstream target genes of STAT3 contribute to malignant progression. To date, a small number of genes have been shown to be regulated by STAT3 including cyclin D1, p21WAF1, Bcl-x, Mcl-1 and c-Myc, which are important in cell cycle control and apoptosis. However, it is likely that additional STAT3-regulated genes participate in oncogenesis. Using Affymetrix microarray chip technology, we have defined a list of genes that are associated with STAT3 activation in cells transformed by the Src oncoprotein and in model human breast carcinoma cell lines. Identification of altered gene expression attributed to STAT3 was accomplished by examining multiple cell lines with different levels of STAT3 activity. The list was refined by altering the STAT3 activation status of various cell lines using a number of approaches, including pharmacologic inhibitors of STAT3 signaling. A range of statistical approaches was employed to identify genes that most accurately correspond to STAT3 activity in the context of oncogenesis. These studies define a molecular fingerprint of candidate STAT3-regulated genes that potentially contribute to malignant progression of breast cancer.

B. (Oncogene Meeting Abstract) Molecular Fingerprint of STAT3 Regulated Genes Associated with Src Oncogenesis and Breast Cancer as Defined by Microarray Technology

Dominic Sinibaldi, Roy Garcia, Greg Bloom, Peter Geiser, Susan Minton, Carlos Muro-Cacho, Emmanuel Lazaridis, and Richard Jove. H. Lee Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine, Tampa, FL 33612.

Signal Transducers and Activators of Transcription (STATs) are latent cytoplasmic transcription factors that are involved in normal cytokine and growth factor signaling. Recent studies have shown that certain STAT family members, most notably STAT3, are constitutively activated by various oncoproteins such as

Src. Furthermore, STAT3 signaling is constitutively activated with high frequency in diverse human tumors including breast carcinoma, multiple myeloma and various leukemias. Expression of dominant-negative forms of STAT3 block cell transformation by the Src oncoprotein, demonstrating that STAT3 has an essential role in oncogenesis. In addition, dominant-negative STAT3 induces apoptosis and cell cycle arrest in model human tumor cell lines such as breast carcinoma. These findings indicate that downstream target genes of STAT3 contribute to malignant progression. To date, a small number of genes have been shown to be regulated by STAT3 including cyclin D1, Bcl-x, Mcl-1 and c-Myc, which are important in cell cycle control and apoptosis. However, it is likely that additional STAT3-regulated genes participate in oncogenesis. Using Affymetrix microarray chip technology, we have defined a list of genes that are associated with STAT3 activation in cells transformed by the Src oncoprotein and in human breast carcinoma cell lines. Identification of altered gene expression attributed to STAT3 was accomplished by examining multiple cell lines with different levels of STAT3 activity. The list was refined by altering the STAT3 activation status of various cell lines using a number of approaches, including inhibitors of STAT3 signaling. A range of novel statistical methods was employed to identify genes that most accurately correspond to STAT3 activity in the context of oncogenesis. These studies define a molecular fingerprint of candidate STAT3-regulated genes that potentially contribute to Src oncogenesis and malignant progression of breast cancer.

# **A Simple Method to Improve Probe Set Estimates from Oligonucleotide Arrays**

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## ***Abstract***

A popular commercially available oligonucleotide microarray technology employs sets of 25 base pair (bp) oligonucleotide probes for measurement of gene expression levels. A mathematical algorithm is required to compute an estimate of gene expression from the multiple probes. Previously proposed methods for summarizing gene expression data have either been substantially ad hoc or have relied on model assumptions that may be easily violated. Here we present a new algorithm for calculating gene expression from probe sets. Our approach is functionally related to leave-one-out cross-validation, a non-parametric statistical technique that is often applied in limited data situations. We illustrate this approach using data from our study seeking a molecular fingerprint of STAT3 regulated genes for early detection of human cancer.

## 1. Introduction

Microarray experiments seek to simultaneously quantitate the relative concentrations of the thousands of messenger RNA (mRNA) molecules in one or more biological samples. The technology promises scientists a detailed view of one level of complex biological systems. Research studies may use biological samples from a variety of sources including human tissue, plant material, and cell cultures. One application is in the area of human cancer where identification of a molecular fingerprint would provide valuable information for the early detection and design of treatment modalities for a variety of tumors. Signal Transducers and Activators of Transcription (STATs) are latent cytoplasmic transcription factors that are involved in normal cytokine and growth factor signaling [1]. STAT3 signaling is constitutively activated with high frequency in diverse human tumors including breast carcinoma, head and neck squamous cell carcinoma, multiple myeloma, and various leukemias. These findings indicate that downstream target genes of STAT3 contribute to malignant progression. To date, a small number of genes have been shown to be regulated by STAT3 including cyclin D1, p21WAF1, Bcl-x, Mcl-1 and c-Myc, which are important in cell cycle control and apoptosis. It is likely that additional STAT3-regulated genes participate in oncogenesis. Microarray technology can be used in an experiment to examine multiple cell lines with various levels of STAT3 activity, thereby identifying a much larger list of STAT3-regulated genes. By altering the extent of STAT3 activation in these cell lines using a variety of approaches, such as pharmacologic inhibitors of STAT3 signaling, the list can be further refined. Thus, a molecular fingerprint of candidate STAT3-regulated genes that may potentially contribute to malignant progression of breast cancer is defined. We employ this approach in our study, "Molecular Fingerprint of STAT3 Regulated Genes for Early Detection of Human Cancer". Data from this study will be considered below.

The term microarray instance refers to a single image of a particular hybridized microarray chip, slide or filter. Here we consider an issue arising in application of a proprietary technology by Affymetrix [3]. The Affymetrix Gene Chip system works by creating a defined array of specific oligonucleotides, or oligos, on a solid support via appropriate sequencing of masks and chemicals in a photolithographic process (similar to that used to manufacture semiconductors). A Biotin-tagged cRNA sample is washed over the chip and hybridized to the complementary oligo probes. A laser then scans the chip and the fluorescence of each probe is measured. The Affymetrix approach uses a designed probe set (typically 40 probes, also called features) of 25 bp oligos per probed gene, which must be algorithmically combined in order to estimate gene expression.

Thus, a special requirement of oligonucleotide chips is a robust method to combine the measured average intensities of oligo features in a probe set into a single value. Typically, each probe set is composed of 40 features, arranged in 20 perfect match – mismatch oligo pairs. Each perfect match oligo is a representative portion of the gene of interest. Each mismatch feature contains a single base pair mutation in the center of the

strand relative to the perfect match. Affymetrix includes mismatch features on chips, intended to provide a means for quality control prior to and during quantitation.

The current software provided by Affymetrix returns the average probe intensities for a subset of features in each probe set. The subset is chosen in each array by considering the mean differences between paired perfect match and mismatch average intensities, after excluding the maximum and the minimum. If probe pair differences deviate by more than 3 standard deviations from the mean, they are excluded in gene expression estimates. In the comparison of two microarray instances, the intersection of acceptable probes in each instance is employed to evaluate gene expression difference. Not only does this procedure potentially exclude informative probes with large responses in individual arrays, it also implicitly assumes that the information provided by each acceptable oligo is of equal importance. A superior alternative to this approach would be a weighted sum of oligo-specific values with parameters chosen to reflect the amount of information in each oligo. (We note that Affymetrix announced a forthcoming version of their analysis software in August, 2001. Unfortunately, this software and its documentation were not available in time for the final revision of this manuscript.)

A procedure for choosing such weights has appeared in the literature. Li and Wong [2] assume that the average intensity of each probe in a probe set increases linearly with respect to increases in underlying, unknown gene expression, but with probe-specific sensitivity. This assumption leads to weighted sum conditional least squares estimates of gene expression. Let  $k$  index genes or probe sets,  $i$  index probes within probe set  $k$ , and  $j$  index array instances. Ignoring the authors' use of mismatch feature intensities, their basic model can be written  $y_{ijk} = \phi_i \theta_{jk} + \varepsilon_{ij}$ , with  $\varepsilon_{ijk} \sim N(0, \sigma^2)$  and  $\sum_i \phi_i^2 = N_k$ ,  $N_k$

being the number of probes in probe set  $k$ . Least squares estimation is performed by iteratively calculating the microarray instance-specific parameters for a particular gene ( $\theta_{jk}$ ) and the associated probe-specific weights ( $\phi_i$ ). In application, this process is enhanced by somewhat ad hoc procedures for identifying and excluding outlier microarray instances and probes (outliers relative to the model) as well as probes with high leverage (which may be untrustworthy because of their influence on the model estimates). Possible drawbacks to this approach arise from reliance on the parametric model, its distributional assumptions, and on the criteria one employs to exclude outlying or untrustworthy data.

## 2. Methods

We employ a non-parametric approach to weighing oligo features using a minimum risk criterion, an approach that is easily described and implemented. Without loss of generality, consider a single probe set. One can think of oligos in this probe set as players on a team selected on evidence that as individuals they are top performers. The performance of any given player can be gauged according to how well that player

estimates a value of interest, in this case, the expression of the gene associated with the probe set. Each microarray instance in a particular analysis corresponds to a single game, resulting in a score for each of the members of the team. Our main question involves a coaching decision, whereby the analyst (coach) seeks to obtain a better estimate of (unobserved) gene expression in each sample,  $\theta_j$ , for a particular probe set.

Formally, let  $i$  index  $N$  players and  $j$  index  $J$  games. Denote the response (average intensity) of each player (oligo) at each game (microarray instance) by  $y_{ij}$ . It can easily be shown that a coach should use  $\bar{y}_{\cdot j}$  to estimate  $\theta_j$  in the situation where all the players perform equally well across games. Let  $\phi_i y_{ij}$  be the coach's estimate of  $\theta_j$ ,  $\phi_i$  being a parameter that corresponds to the coach's evaluation of player  $i$ . Values of  $\phi_i$  near 1 suggest that the coach believes player  $i$  to be a good unbiased estimator of the  $\theta_j$  over the  $J$  games. Values larger than 1 suggest that the coach believes player  $i$  to be a low biased estimator of  $\theta_j$ . Values near 0 suggest that the coach believes player  $i$  either estimates  $\theta_j$  too high, or perhaps, that player  $i$  is so inconsistent over games as to require this player's contribution be highly discounted.

In the microarray context, relative performance of players is unknown a priori and must be estimated by the coach. Since the values of  $\theta_j$  are unknown, exact loss functions cannot be calculated. Instead, it is reasonable to suppose that the rational coach should evaluate each player against a best estimate of gene expression derived from the rest of the team.

Assuming equal importance of games played, the problem of minimizing loss over players and games can be addressed by calculating a set of parameters,  $\phi_i$ , such that a sum of squares loss over players and games

$$S(\phi; y) = \sum_i \sum_j \left( \phi_i y_{ij} - \frac{\sum_{k: k \neq i} \phi_k y_{kj}}{\phi_{\cdot} - \phi_i} \right)^2$$

is minimized. This procedure may be applied to each probe set on one or more microarray instances. The  $\phi_i$  parameters are not normalized in our procedure.

Minimization can be performed using conjugate gradient methods available in many packages. After prototyping in MathCAD, we employed the `nlminb()` function in S-Plus to minimize this expression in terms of  $\phi$ . Because of speed issues in S-Plus, we are presently testing a C routine to minimize this function. We have also tested `proc nlp` in the SAS System with excellent results.

### 3. Results

We illustrate application of this approach using data from our study, "Molecular Fingerprint of STAT3 Regulated Genes for Early Detection of Human Cancer", described above. As part of this study we are comparing cells expressing activated STAT3 under various conditions to cells not expressing activated STAT3. A set of 6 microarray instances, derived using the HuGeneFL array (Affymetrix), were analyzed for transcriptional changes related to STAT3 expression. The data for a particular gene of interest to us are shown in the bulk of Table 1.

We applied our new algorithm to probe sets across these 6 instances. Table 1 gives our estimated values of  $\phi_i$  for each oligo in this probe set in the second column from the right. Using these values, we estimated gene expression, and compared these estimates to the corresponding mean and median of the probe set for each microarray instance. These values are shown towards the bottom of Table 1. In addition, we applied the Wong algorithm to our perfect match data, without their ad hoc criteria for reducing the influence of outlying observations. The corresponding probe weights and gene expression estimates are given in Table 1. Finally, for reference we report the Affymetrix average difference values, and Wong estimates using perfect match and mismatch data.

### 4. Discussion

The estimated values of  $\phi_i$  for our method in Table 1 demonstrate that individual probes in a single probe set range almost 20-fold in performance over the 6 microarray instances in this analysis.

While both our method and the Wong approach agree that the probes in this probe set are far from equivalent in terms of information content, the weight estimates from the two approaches are radically different. Note that there are 5 probes with very large average intensities (probes 1,5,8,13 and 15) over microarray instances 4 through 6. These probes are heavily weighted by the Wong model, whereas they are substantially discounted by our approach. All of these probes are influential in the Wong conditionally linear model, but not to the degree that they would be excluded by the recommended criteria for outlier identification. Since together they provide a full quarter of the data, their effect on the Wong model estimates of gene expression is sizable. Our method places more trust in the remaining 15 probes, which demonstrate less radical changes in gene expression in the last three instances. This agrees with our assumption that all the players on a particular probe set team are pros at the task they are intended to perform. Consequently, Table 1 illustrates that our gene expression estimates are even less influenced by the large values in instances 4 to 6 than are the sample medians.

Formally, our procedure is equivalent to minimizing the leave-one-out cross-validation estimate of variance for the mean of coach-adjusted player estimates,  $\phi_i y_{ij}$ , for

each probe set. In conjunction with the above example, this rationale suggests that our approach may outperform parametric methods especially in settings where limited information is available about probe weights. For example, designed laboratory experiments typically result in few microarray instances over a multiplicity of conditions. Situations in which conditions of a linear model with constant variance may be violated will often arise in laboratory experiments due to the hybridization performance of different molecular mixtures across samples being compared. Furthermore, when each microarray instance represents a specific biological condition in a designed experiment (as opposed to a human tumor sample), it is not reasonable to exclude data based on statistical reasoning alone.

We note that the existence of mismatch oligos in each probe set on an Affymetrix chip is something of a white elephant. It is possible to run algorithms on perfect-match to mismatch pair differences; however, it is not clear what benefit this could give to inference since some molecules with high affinity to a mismatch oligo may have low affinity to the corresponding perfect match. This fact is ignored by Li and Wong, and indeed, by Affymetrix. We illustrate this problem in Figure 1.

Even without mismatch oligo information, our method properly discounts oligo values that are too high since perfect-match oligos with large average intensities are the most likely candidates for non-specific hybridization. When many perfect-match oligo probes are brightly lit, a brightly-lit probe with a bright mismatch partner should not necessarily be discounted. If its perfect match values across microarray instances agree with many other members of the probe set, it may be that the molecules hybridizing to the mismatch probe are not similarly hybridizing to the perfect match.

One must have gold standard estimates in order to determine which technique is best for estimating true gene expression in an unbiased and minimum variance fashion. As part of our collaborative project, we are completing real time PCR assays on a large set of genes to provide the necessary standard for testing among probe set gene expression models. With this information we will be able to benchmark the biological performance of multiple algorithms.

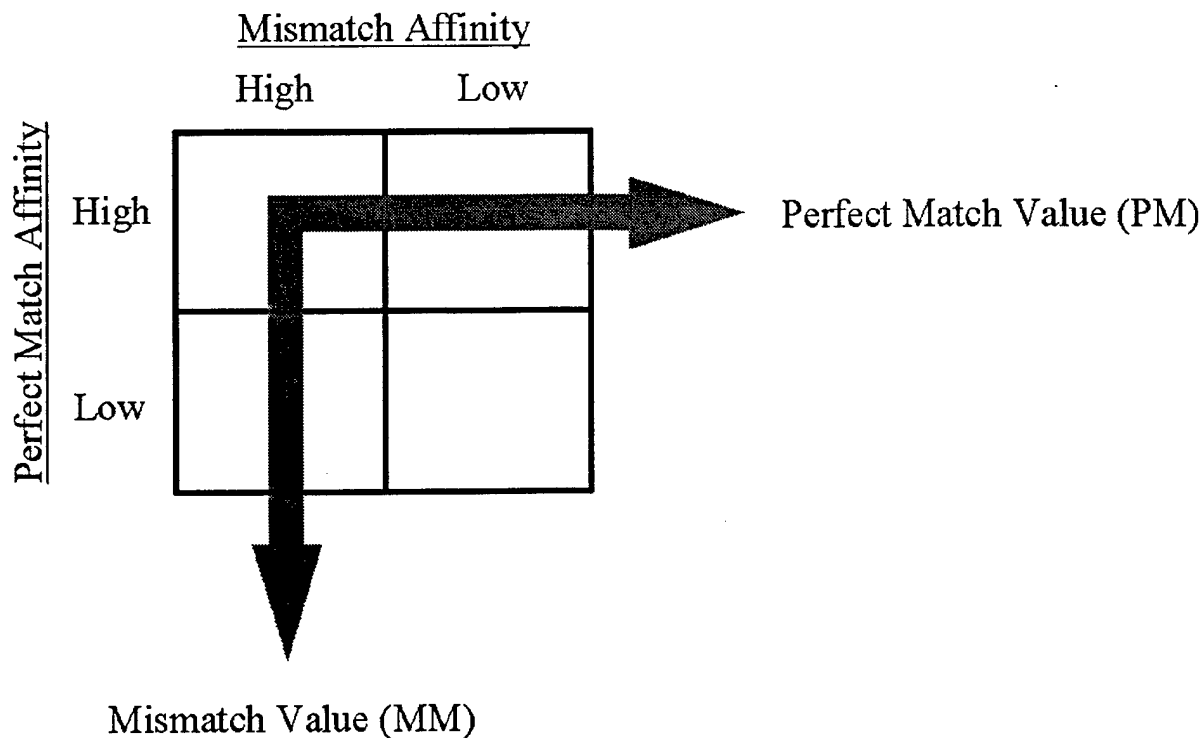
#### References

- [1] Bowman T., Garcia R., Turkson J. and Jove R., STATs in oncogenesis. *Oncogene* 19 (2000) pp. 2474-2488.
- [2] Li C. and Wong W. H., Model-based analysis of oligonucleotide arrays: Expression index computation and outlier detection. *Proc. Nat Acad. Sci. USA* 98 (2001) pp. 31-36.
- [3] Lockhart D., Dong H., Byrne M., Follettie M., Gallo M., Chee M., Mittmann M., Wang C., Kobayashi M., Horton H., et al., *Nat. Biotech.* 14 (1996) pp. 1675-1680.

Table 1: Illustrative Data and Results of Applying the New Algorithm to Perfect-Match Average Intensities of Probes in a Particular Probe Set of Interest

Oligo	Microarray Instance						$\phi_i$	
	j=1	2	3	4	5	6	New	Wong
i=1	275.24	261.84	317.16	918.43	2194.13	2261.82	0.14	2.14
2	310.63	84.81	91.79	656.00	631.90	643.72	0.48	0.75
3	356.95	89.90	88.32	817.74	606.64	563.94	0.46	0.76
4	265.96	59.64	63.84	238.13	175.65	177.95	1.53	0.25
5	499.97	194.34	186.17	1618.11	1536.75	1773.39	0.18	1.89
6	252.15	55.13	58.56	176.25	60.66	89.15	1.99	0.15
7	230.50	51.99	64.20	255.65	73.07	246.36	1.51	0.24
8	293.73	95.75	93.34	1307.93	1026.25	1734.76	0.21	1.56
9	243.28	66.18	73.13	393.20	277.20	344.73	0.97	0.40
10	257.82	69.17	70.49	445.05	406.65	437.28	0.76	0.51
11	303.01	126.65	109.28	582.89	932.66	888.91	0.36	0.95
12	256.79	59.20	70.36	236.48	185.22	229.24	1.46	0.27
13	314.00	93.37	98.98	668.26	1165.54	1325.71	0.26	1.25
14	466.30	162.62	143.41	691.73	527.25	465.18	0.52	0.67
15	265.55	72.04	86.85	1100.86	1888.96	1878.34	0.16	1.89
16	277.30	61.74	65.13	604.85	697.87	668.94	0.47	0.77
17	251.29	56.45	59.15	165.33	73.85	82.39	2.03	0.14
18	253.18	56.79	54.53	190.49	97.32	99.61	1.93	0.17
19	334.00	96.01	98.62	448.77	678.74	555.37	0.54	0.68
20	285.03	75.59	87.19	228.30	104.49	113.35	1.56	0.18
Avg:	299.63	94.46	99.02	587.22	667.04	729.01		
Median:	276.27	73.81	87.02	515.83	566.95	510.28		
New:	273.68	71.02	75.25	345.23	293.10	327.73		
Wong:	251.25	97.49	103.80	878.19	894.53	983.06		

Figure 1: Paired Match and Mismatch Probes Are Observations on the Margins of a 2 x 2 Table



A third observation with high-high or high-low affinity is not experimentally available, precluding use of the mismatch value as an internal control. We note that across the studies we have analyzed to date, 22% of match cells with mismatch above background are less bright than their mismatch partner.



# **APPENDIX F**

## **Tampa Bay Ovarian Cancer Study**

**Rebecca Sutphen, M.D.**

Tampa Bay Ovarian Cancer Study  
Principal Investigator: Rebecca Sutphen, M.D.

1. Introduction

The BRCA1 and BRCA2 genes are believed to account for the majority of inherited ovarian cancer, yet few population-based studies have been performed specifically to investigate their role in this deadly disease. The Tampa Bay Ovarian Cancer Study (TBOCS) is a case-case study of incident epithelial ovarian cancer in the geographic region of Hillsborough and Pinellas counties, Florida, comprising an estimated 170 annual cases in women between 18 and 80 years of age. A rapid ascertainment system is utilized. In-person interviews are conducted with all subjects, in order to collect comprehensive data on health behaviors, risk factors, personal and family history; provide genetic counseling; and obtain blood samples. Complete sequencing of the BRCA1 and BRCA2 coding regions is performed to allow assignment of cases (mutation-carriers) and controls (non-carriers); we will thereby determine the prevalence of BRCA1 and BRCA2 germline mutations in this population. The purpose of this initial project is to demonstrate the feasibility of conducting a 5-year successor study of ovarian cancer cases associated with germline BRCA1/BRCA2 mutations, compared with sporadic cases. The specific aims of this feasibility study and the expanded successor study of incident epithelial ovarian cancer are:

- 1) to investigate whether and which health behaviors and risk factors differ between germline mutation-associated cases and non-mutation controls;
- 2) to examine differences in the family cancer history profile of mutation-associated cases and non-mutation controls;
- 3) to examine differences in tumor characteristics between mutation-associated cases and non-mutation controls;
- 4) to investigate differences in response to treatment and survival between mutation-associated cases and non-mutation controls;
- 5) to achieve an 80% participation rate using a unique and comprehensive recruitment design.

2. Body

The study was reviewed by the Surgeon General's Human Subjects Research Review Board (SGHSRRB) on September 27, 2000. Final approval to open the study for enrollment was obtained on December 13, 2000.

Status of tasks included in the approved statement of work are as follows:

Task 1: Preparation for Medical Record Abstractions

Data elements of the medical record abstraction form have been finalized. Design of the medical record abstraction form and the data entry mechanism for pathology data has been completed. This design allows direct data entry of abstracted medical records into the database, followed by independent review of the medical record data by the pathologist at the time of pathology analysis. This mechanism makes data entry highly efficient while ensuring data quality.

## Task 2: Recruitment and Training of Study Personnel

The first recruitment strategy to be implemented involved recruiting all gynecologic oncologists in the region as co-Investigators in the study and training their staff regarding the study. At this time, this first strategy has been accomplished, except that one of the 7 gynecologic oncologists requires additional strategies (described further below) to accomplish recruitment. Additional study personnel are currently being trained. Other recruitment strategies are currently being implemented, including: 1) \$100 reimbursement per enrollment is planned to the physician offices to compensate for time, effort and space allocated to this project. This strategy has received USF IRB approval and is being reviewed by the Surgeon General's Research Review Board (submitted June 11, 2001) prior to implementation. 2) recruitment through medical oncologists' offices is being implemented to accrue subjects missed at the gynecologic oncologists' offices, and additional co-Investigators have been added, 3) institutional IRB approval is being obtained at diagnosing hospitals in the study region to allow study staff to obtain names of patients with ovarian cancer so that study staff may assume responsibility to contact ovarian cancer patients through a letter signed by the staff physician, and follow up via phone call to determine interest in participation. By centralizing responsibility for patient contact to study staff, we will decrease responsibilities of local physicians' staff, which will facilitate enrollment and enhance accrual. Approval from the USF IRB for this strategy has been requested. Approval from the Surgeon General's Research Review Board will also be required.

## Task 3: Subject recruitment and Data Collection

Based on experience thus far, the study interview appears to be a successful strategy to accomplish explanation of the study, provision of informed consent, enrollment, completion of study questionnaire, genetic counseling, and blood sampling. Medical records have been successfully obtained for all recruits and abstraction performed. Tumor tissue has been successfully obtained from the appropriate surgical locations for all pathology analyses. Blood samples have been successfully obtained for genetic testing and banking for future research.

## Task 4: Disclosure of results to the patients

The results of genetic testing and related information (depending on results) have been provided to subjects who elect to receive results. Results of genetic testing have been utilized for assignment of case-control status and matching.

## Task 5: Abstraction of Medical Records

Medical record abstractions have been performed for all enrolled subjects. 6 month follow-up data has been obtained for appropriate participants. Data entry and quality control measures are ongoing.

## Task 6: Tumor Tissue Analyses

Tumor tissue has been collected for all subjects. Pathology analyses will be performed, data from analyses will be entered into the database, and tissue will then be banked for future research.

#### Task 7: Follow-up for Survival

Six month follow-up data has been obtained as appropriate. Follow-up contacts will continue for surviving enrolled subjects after 6 months, after 1 year, and annually as funding permits. Data entry and quality control measures are ongoing.

#### Task 8: Statistical Analyses and report writing

Analyses and reports will be prepared.

### 3. Key Research Accomplishments

Aim 1: we are successfully collecting data regarding health behaviors and risk factors from all participants via questionnaire instruments and study interview.

Aim 2: we are successfully collecting detailed cancer family history from all participants via questionnaire instruments and study interview.

Aim 3: we have established a successful mechanism to obtain medical records and tumor tissue in order to compare tumor characteristics between mutation-associated cases and non-mutation controls.

Aim 4: we have established a successful follow-up mechanism to obtain data regarding differences in response to treatment and survival between mutation-associated cases and non-mutation controls.

Aim 5: we are implementing additional strategies to achieve 80% participation of eligible cases and anticipate success by summer 2002.

### 4. Reportable Outcomes

Based on the epidemiologic design of the Tampa Bay Ovarian Cancer Study, funding was awarded by the American Cancer Society for a 3-year companion study to evaluate the role of biologically active lysophospholipids for their potential as biomarkers of ovarian cancer. Preliminary data is promising and shows that certain lysolipids appear to be elevated in the plasma of women with ovarian cancer compared with healthy controls. Based on this preliminary data, we have applied for an R01 to investigate the use of lysolipid measurement for detection of ovarian cancer in a population of women at increased risk of ovarian cancer, including first-degree relatives of women in TBOCS (ovarian cancer patients). Our ongoing contact with women in TBOCS will facilitate the identification and enrollment of their female relatives at increased risk for enrollment in this important study, toward the development of an early detection test for ovarian cancer.

Based on data showing that gene mutations associated with Hereditary Non-Polyposis Colorectal Cancer (HNPCC) are the third leading cause of hereditary ovarian cancer (after BRCA1 and BRCA2), and the suggestion that ovarian cancer is a "sentinel cancer" in individuals with these gene mutations, funding for the investigation of HNPCC as a companion study of TBOCS has been proposed.

## 5. Conclusions

Epithelial ovarian cancer results in the death of more American women than all other gynecologic cancers combined. The Tampa Bay Ovarian Cancer Study is designed to evaluate the role of the BRCA1 and BRCA2 genes in the etiology, pathology and response to treatment of this deadly disease. Additional recruitment strategies are being implemented to achieve enrollment of 80% of eligible ovarian cancer patients. Current funding supports the initial development of a successful accrual strategy toward a planned 5-year successor study. Based on the design of the feasibility analysis and successor study, a 3-year companion study has been funded to assess the potential of biologically active lysophospholipids as biomarkers in ovarian cancer. The Tampa Bay Ovarian Cancer Study and its companion study represent an important opportunity to evaluate the role of inherited susceptibility to ovarian cancer and evaluate lysophospholipids for their potential as biomarkers of this deadly disease. No similar study has been performed. In order to accomplish these critical goals, continued funding of the current study is required.

# **APPENDIX G**

## **Molecular Predictors of Disease Behavior in Thyroid Cancer**

**Carlos Muro-Cacho, M.D., Ph.D.**

# ANNUAL PROGRESS REPORT – NOVEMBER 2001

Proposal Title: **Molecular Predictors of Disease Behavior in Thyroid Cancer**  
Primary Investigator: **Carlos A. Muro-Cacho, MD, PhD**

## (1) INTRODUCTION

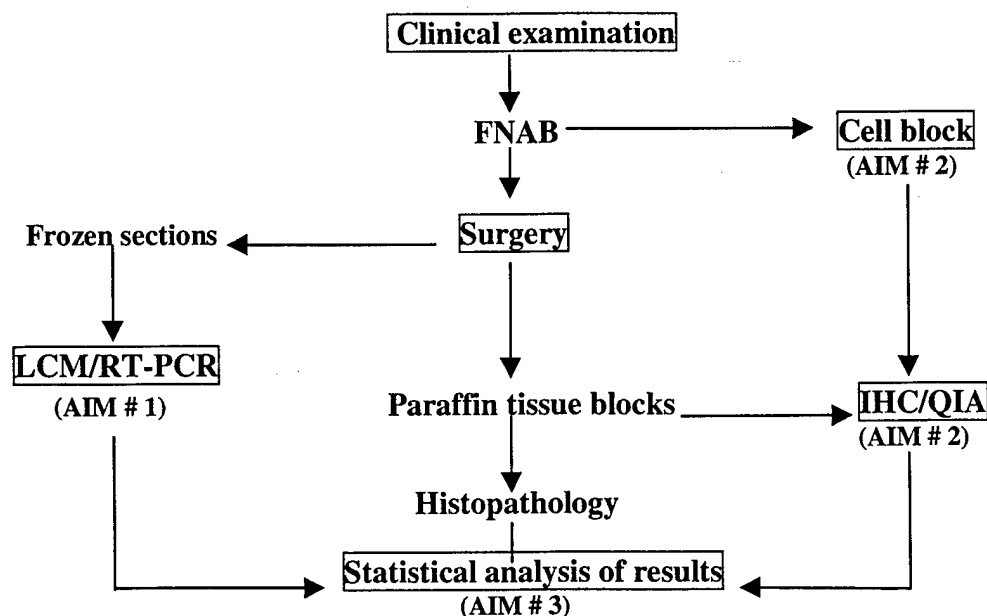
**SUBJECT.** Clinico-pathological parameters are insufficient to predict the behavior of papillary carcinomas of the thyroid gland. Therefore, molecular predictors of prognosis are desperately needed to provide an individualized approach to treatment. In thyroid tumors, oncogenesis is in part related to an acquired resistance to the normal growth inhibitory control mediated by Transforming Growth Factor  $\beta$  (TGF $\beta$ ). Also, RET translocations have been reported to occur in thyroid cancer, but their true incidence, clinical significance, mechanisms of action, and relationship to other pathways are unknown.

**PURPOSE.** Our hypothesis is that abnormalities of the TGF $\beta$  and RET pathways are important in thyroid oncogenesis and represent ideal candidates in the search for molecular predictors. The overall objective of this proposal was, therefore, to identify molecular prognostic information that could be incorporated prospectively in the management algorithm of thyroid cancer.

**SCOPE.** To validate the prospective use of this molecular information, we had proposed to investigate TGF $\beta$  pathway and RET abnormalities, at the RNA and protein levels, in surgically resected thyroid carcinomas, and to correlate these findings with those obtained in tumor cells obtained prior to surgery. Ultimately, these results would be statistically correlated with clinico-pathological parameters to identify molecular prognostic parameters of the disease.

## (2) BODY

In the initial proposal the following working algorithm was proposed:



LCM = Laser MicroDissection; RT-PCR: Reverse Transcriptase Polymerase Chain Reaction; FNAB = Fine Needle Aspiration Biopsy; IHC = Immunohistochemistry; QIA = Quantitation by Image Analysis  
The three Specific Aims were described as follows:

***SPECIFIC AIM # 1 - To analyze RET translocations and abnormalities of the TGF $\beta$  pathway in surgically resected thyroid carcinomas.*** Frozen sections will be prepared from tumors immediately after surgical removal. Laser Capture Microdissection will be used to independently isolate RNA from tumor cells and adjacent non-neoplastic thyroid tissue. RT-PCR will be performed to detect mRNA of the molecules of interest in both cell populations, separately.

***SPECIFIC AIM # 2 - To investigate by immunohistochemistry (IHC) the expression of members of the TGF $\beta$  pathway in tumor cells obtained prior to surgery and in tissue sections of the same tumors after surgery.*** Tumor cells obtained by Fine Needle Aspiration Biopsy (FNAB) will be analyzed by IHC for the presence of molecular abnormalities. Results will be correlated with information obtained from the analysis of the same tumors after surgery.

***SPECIFIC AIM # 3 - To statistically correlate results from Specific Aims # 1 and #2 with clinico-pathological parameters to identify molecular prognostic parameters of the disease.*** The studies described in Specific Aims 1 and 2 will allow the comparison between tumor and adjacent non-neoplastic tissue and between tumor pairs, pre- and post-surgery. Results from these "prospective" (FNAB) and "retrospective" (tumors surgically removed) will be interpreted and statistically analyzed in the context of clinico-pathological parameters.

## **PROGRESS TO DATE**

### **SPECIFIC AIM # 1**

In the initial proposal, this Specific Aim depended on appropriate collection of tissues following specific protocols.

**Collection of tissues.** In the initial proposal, it was estimated that approximately 60 papillary carcinomas could be collected for the purpose of this study. This estimate was based on:

- a.) Accumulated data from the previous five years on the number of thyroid surgeries and levels of operating-room utilization.
- b.) Projected volumes in thyroid surgery obtained from the Head and Neck Program and operating-room expansions already initiated by the Surgery Service.

Two important and unexpected events, however, have complicated the collection of tissues for this study since the initial proposal was submitted.

- a.) The unexpected departure from the institution of the surgeon specialized in thyroid surgery has made the initial goal of accruing 60 cases in a year impossible to achieve. As a result, only 10 carcinomas suitable for analysis have been collected to date. Also, 3 follicular adenomas and 5 nodular hyperplasias have been collected as non-malignant and non-neoplastic controls, respectively. In all these cases, tumor and normal tissue counterpart was collected following the protocols described in the proposal (see below).



b.) Second, the changes in managerial approaches to thyroid cancer implemented by the new surgical team have limited the role of pre-surgical fine-needle aspiration biopsy (FNAB). This, together with the also unexpected departure from the Pathology Department of a cytopathologist specialized on FNAB of the thyroid gland, has seriously limited the availability of cytological material for correlative studies with tissue counterparts. In the period of time when the proposal was active, a total of 30 thyroid FNAs were performed. Of those, only 5 contained sufficient material to allow cell block preparation and subsequent analysis.

Certain modifications of the initial proposal are recommended (see below) to partially overcome these difficulties:

1. Correlative analysis of immunohistochemistry data in tissue and cytological preparations could be performed on approximately 20 selected archival cases.
2. RET translocation analysis could be performed on DNA extracted from the paraffin blocks of the same cases (this method has been successfully used in our laboratory to detect DNA for other purposes).

**Sample Preparation.** In each of the cases collected, 10 consecutive frozen sections were prepared within 10 minutes from the time blood flow was surgically interrupted. These frozen sections were image counterparts of the tissue used for immunohistochemistry after formalin fixation and embedding in paraffin. One of the frozen sections was stained with hematoxylin-eosin to identify selected normal and tumor areas. Laser Capture Microdissection was used to independently isolate DNA and RNA from tumor cells and adjacent non-neoplastic thyroid tissue (See Appendix, Figure 1). It was determined that 500 laser hits were necessary to obtain sufficient DNA for analysis while at least 1000 laser hits were necessary to obtain sufficient RNA.

The conditions for PCR using the selected primers have been optimized for members of the TGF- $\beta$  pathway (see Appendix, Figures 2 and 3).

The sequences of all primers for the three more important RET translocations (see below) were obtained from pertinent publications and confirmed with GeneBank data. The corresponding primers were constructed and extensively tested using tumor tissue and cell lines, and a variety of PCR conditions. The bands extracted from the gels are similar in size to the published bands. After sequencing the bands extracted from the gels, however, none of the sequenced fragments has proven to correspond to the published sequences. We are currently in the process of investigating these results.

Translocation	Primer combination
Ret/PTC1	S: 5' ATTGTCATCTCGCCGTTTC 3' AS: 5' CTTTCAGCATCTTCACGG 3'
Ret/PTC2	S: 5' GAGGGAGCTTTGGAGAAC 3' AS: 5' CTTTCAGCATCTTCACGG 3'
Ret/PTC3	S: 5' AAGCAAACCTGCCAGTGG 3' AS: 5' CTTTCAGCATCTTCACGG 3'

## SPECIFIC AIM # 2

Immunohistochemistry conditions for the members of the TGF- $\beta$  pathway have been optimized and all cases collected to date have been stained for all markers. Conditions for image acquisition, calibration, and automatic measurement have been established using the Optimas 6.5 software and a Spot digital camera and

software. A change to initial planning has been the use of a red chromogen (Nova Red) instead of the common brown diaminobenzidine. This has proven to enhance discrimination of positive red signals versus the blue hematoxylin counterstaining. Preliminary studies of staining heterogeneity have revealed minimal changes in quantitation values within the thickness of tissue corresponding to 10 consecutive slides 4 micron each, well within the scope of current experiments. Quantitation of protocol cases will begin soon (See Appendix, Table 1).

### **SPECIFIC AIM # 3**

Statistical analysis has not been performed as yet. This will be attempted once the image analysis data has been generated.

### **(3) KEY RESEARCH ACCOMPLISHMENTS**

Nothing to report until full analysis is done.

### **(4) REPORTABLE OUTCOMES**

None at this time.

### **(5) CONCLUSIONS**

The analysis of the cases accumulated so far will be done shortly. The study will focus on the members of the TGF- $\beta$  pathway. The five cases where cytologic material is available will also be study for the most promising markers. If the protocols for RET translocation analysis are optimized, this analysis will be done immediately afterwards.

### **(6) REFERENCES**

None at this time.

## (7) APPENDIX

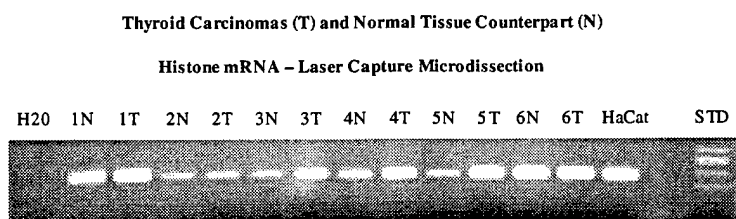


Figure 1. Amplification of histone mRNA after Laser Capture Microdissection

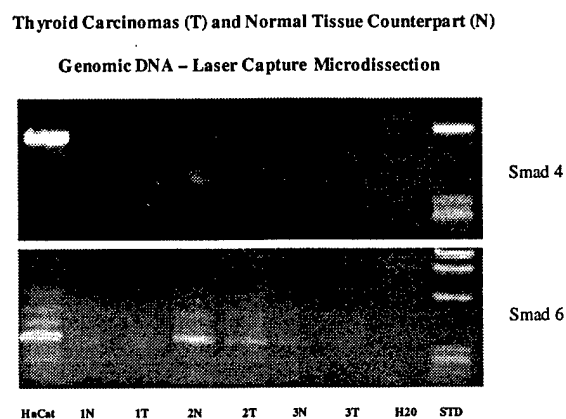
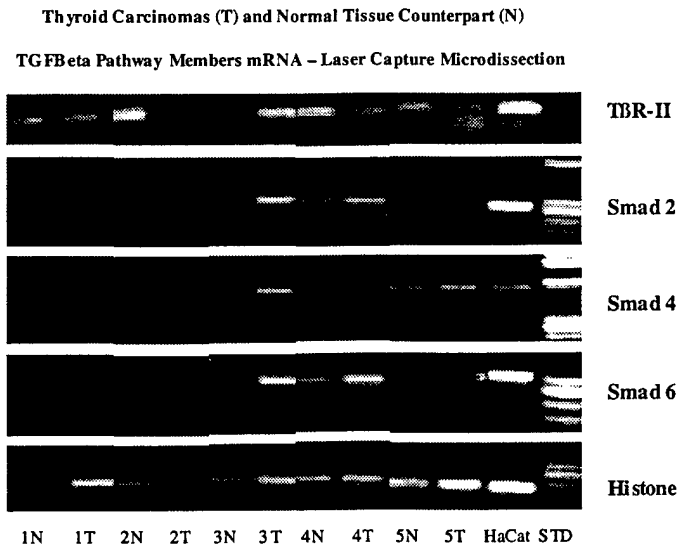


Figure 2. Amplification of Smad4 and Smad 6 from genomic DNA extracted from the tissue by Laser Capture Microdissection



**Figure 3. Amplification of the mRNAs of TGF- $\beta$  pathway members by RT-PCR from material obtained by Laser Capture Microdissection**

Case	mRNA Analysis (RT-PCR)						Immunohistochemistry						RET Analysis
	Histone	Smad 2	Smad 4	Smad 3	Smad 6	TβRII	Smad2	P-Smad2	Smad4	Smad 3	Smad 6	TβRII	
1	N +	-/+	+	+	-/+	+	Done	Done	Done	Done	Done	Done	Pending
	T +	-/+	+	+	-/+	+	Done	Done	Done	Done	Done	Done	Pending
2	N +	+	-	+	+	+++	Done	Done	Done	Done	Done	Done	Pending
	T +	-	-	+	-	-	Done	Done	Done	Done	Done	Done	Pending
3	N +	+	+	+	+	+	Done	Done	Done	Done	Done	Done	Pending
	T +	++	+	+	+++	+	Done	Done	Done	Done	Done	Done	Pending
4	N +	+	+	+	+	+++	Done	Done	Done	Done	Done	Done	Pending
	T +	+	+	+	+++	+	Done	Done	Done	Done	Done	Done	Pending
5	N +	+	+	+	++	+	Done	Done	Done	Done	Done	Done	Pending
	T +	-	+	+	+	+	Done	Done	Done	Done	Done	Done	Pending
6	N +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
	T +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
7	N +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
	T +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
8	N +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
	T +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
9	N +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
	T +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
10	N +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
	T +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
11	N +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
	T +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
12	N +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
	T +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
13	N +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending
	T +	Pending	Pending	Pending	Pending	Pending	Done	Done	Done	Done	Done	Done	Pending

Table 1. RT-PCR in cases 6 to 13 pending. Image Analysis Quantitation on immunohistochemistry slides pending. RET Analysis pending

## **APPENDIX H**

### **Significance of Bax-Dependent Apoptosis in Prevention and Detection of Human Prostate and Lung Cancer**

**Q. Ping Dou, Ph.D.**

## ANNUAL REPORT

**Report Date:** 10-17-2001

**Proposal Title:** *Significance of Bax-Dependent Apoptosis in Prevention and Detection of Human Prostate and Lung Cancer*

**PI's Full Name:** Q. Ping Dou

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**Proposal start date:** 10/01/00 - 9/30/01

### (1) INTRODUCTION:

The ubiquitin/proteasome-mediated protein degradation pathway plays an important role in regulating both cell proliferation and cell death (1-3). Programmed cell death (apoptosis) occurs in two physiological stages, commitment and execution, which are regulated by the Bcl-2 family proteins (4-6) and caspases (7-9), respectively. Decreased Bax expression or Bax/Bcl-2 ratio has been suggested as a predictive marker for metastasis and drug-resistance of human prostate and lung cancers (10-15), whereas increased Bax or Bax/Bcl-2 ratio was associated with prostate and lung cancer cell apoptosis in cultures and animal models (16-20). Therefore, discovery of the molecular mechanism regulating Bax expression has great clinical significance in prevention, detection and treatment of human prostate, lung and other cancers. We have generated the following *THREE HYPOTHESES*. (i) Bax degradation activity (BDA) levels determine Bax levels and therefore predict the progressive stages of prostate and lung cancer. (ii) BDA is also a diagnostic marker for response of prostate and lung cancer cells to proteasome inhibitor-induced apoptosis. (iii) A tumor-specific activity regulating BDA is a putative cancer susceptibility marker. There are *THREE SPECIFIC AIMS* proposed in this grant. Specific Aim 1 is to investigate whether BDA levels determine Bax levels in human prostate and lung cancer cell lines and tissue samples and whether BDA levels correlate cancer progressive stages. Specific Aim 2 is to investigate whether BDA levels predict response of human prostate and lung cancer cells to proteasome inhibitor-induced apoptosis in cultures. Specific Aim 3 is to look for the prostate and lung cancer risk marker that is involved in tumor-specific responsiveness to proteasome inhibitor-induced, Bax-/cytochrome c-dependent apoptosis.

## (2) BODY:

### Prostate Cancer System

In this part of the report, we have presented results generated mainly from human prostate cancer cells. We have used a tea proteasome inhibitor (EGCG) in most of the studies. We have focused on Bax as a mediator of the proteasome inhibitor-induced apoptosis.

**Pharmacological proteasome inhibitors as potential cancer preventative and therapeutic drugs** (21 and see Appendix). The involvement of the ubiquitin/proteasome-dependent degradation pathway in both up-regulation of cell proliferation and down-regulation of cell death in tumor cells suggests the potential use of proteasome inhibitors as novel cancer preventative and therapeutic drugs. This hypothesis has been supported by both *in vitro* and *in vivo* experimental results. *In vitro*, proteasome inhibitors rapidly induce apoptosis in a variety of human cancer cell lines, selectively trigger programmed cell death (apoptosis) in the oncogene-transformed, but not normal or untransformed cells, and are even able to activate the death program in human cancer cells that are resistant to various anticancer agents. *In vivo*, proteasome inhibitors suppress tumor growth *via* induction of cancer cell apoptosis and inhibit the process of angiogenesis. In addition, results from Phase I studies of the proteasome inhibitor PS-341 demonstrates that PS-341 has clinical activity without toxicity, further supporting the hypothesis that proteasome inhibitors can be used as cancer preventative and therapeutic drugs.

**Ester bond-containing tea polyphenols potently inhibit proteasome activity *in vitro* and *in vivo*** (22 and see Appendix). It has been found that proteasome inhibitors are able to induce tumor growth arrest or cell death and that tea consumption is correlated with cancer prevention. We have shown that ester bond-containing tea polyphenols [such as (-)-epigallocatechin-3-gallate or (-)-EGCG] potently and specifically inhibit the chymotrypsin-like activity of the proteasome *in vitro* (IC<sub>50</sub> 86-194 nM) and *in vivo* (1-10  $\mu$ M) at the concentrations found in the serum of green tea drinkers. Atomic orbital energy analyses and high-performance liquid chromatography suggest that the carbon of the polyphenol ester bond is essential for targeting and thereby inhibiting the proteasome in cancer cells. This inhibition of the proteasome by (-)-EGCG in several tumor and transformed cell lines results in accumulation of two natural proteasome substrates, p27<sup>Kip1</sup> and I $\kappa$ B- $\alpha$ , followed by growth arrest in the G<sub>1</sub> phase of the cell cycle. Furthermore, compared to their simian virus-transformed counterpart, the parental normal human fibroblasts were much more resistant to EGCG-induced p27<sup>Kip1</sup> protein accumulation and G<sub>1</sub> arrest. Our study suggests that the proteasome is a cancer-related molecular target of tea polyphenols and that inhibition of the proteasome activity by ester bond-containing polyphenols may contribute to the cancer preventative effect of tea.

**Effects of (-)-EGCG on inhibiting proteasomal chymotrypsin-like activity in prostate cancer cell extracts.** Toward the goal of applying tea polyphenols to human prostate cancer cell systems, we measured potencies of (-)-EGCG in inhibition of the proteasomal activity in prostate cancer cell extracts. The proteasomal chymotrypsin-like activity in prostate cancer PC-3 cell extract was significantly inhibited by (-)-EGCG, whose IC<sub>50</sub> value was calculated to be 14  $\mu$ M (Fig. 1A). When a protein extract of DU145 (Fig. 1B) or LNCaP (data not shown) was used, (-)-EGCG was again found to be a potent inhibitor of the proteasomal chymotrypsin-like activity (with IC<sub>50</sub> values  $\sim$ 6  $\mu$ M). Furthermore, (-)-EGCG had much less inhibitory effects on the trypsin-like activity of the proteasome in PC-3 cell extracts (Fig. 1C). From these data, we conclude that (-)-EGCG is a potent inhibitor of the prostate cancer proteasomal chymotrypsin-like activity.

**(-)-EGCG inhibits the proteasomal activity in intact prostate cancer cells.** We have found (22) that the peptide substrate Suc-Leu-Leu-Val-Tyr-AMC can be used to measure the proteasomal chymotrypsin-like activity in intact PC-3 and Jurkat T cells, which is inhibitable by pre- and co-incubation with (-)-EGCG (Fig.



2). (-)-EGCG at 10  $\mu$ M inhibited ~20% of the chymotryptic activity of the proteasome in living PC-3 cells in this experiment (Fig. 2).

**Treatment of prostate cancer cells with (-)-EGCG accumulates the pro-apoptotic Bax protein.** We and others have found that Bax protein expression is much higher in PC-3 and LNCaP cells than in DU145 cells (23-25; Fig. 3B, lanes 2 vs. 1; Fig. 3C, lanes 1 vs. 2). In addition, (-)-EGCG treatment increased levels of Bax protein in LNCaP (Fig. 3A, lane 4 vs. 1), PC-3 (Fig. 3B, lanes 3 vs. 2), and even DU-145 cells (Fig. 3C, lanes 2-5). However, (-)-EGC at the same or even a higher concentration had no such an effect (Fig. 3A, lanes 2, 3 vs. 4; Fig. 3C, lanes 6 vs. 5). In these experiments, a high molecular weight band(s) (indicated by arrowhead) with unknown nature, which can be recognized by the anti-Bax antibody, is used as a loading control.

In addition, (-)-EGCG at 10 and 25  $\mu$ M significantly increased levels of Bax in LNCaP cells (Fig. 4A). When DU145 cells were used that contain very low basal levels of Bax (see Fig. 3B and C), (-)-EGCG treatment resulted in accumulation of Bax in a concentration-dependent manner (Fig. 4B). In this experiment, levels of actin were measured as a loading control (Fig. 4A and B). These data suggest that (-)-EGCG is able to inhibit the Bax degradation enzyme, the proteasome, *in vivo*. These results also suggest that comparison of PC-3 and LNCaP to DU145 cell lines provides an excellent model for studying the role of Bax in the process of EGCG-mediated proteasome inhibition and apoptosis induction (see below Fig. 5).

**Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in prostate tumor survival and progression** (26). We have shown that inhibition of the proteasome activity in Bcl-2-overexpressing cells accumulates the pro-apoptotic Bax protein in mitochondria/cytoplasm, where it interacts with Bcl-2 protein. This was followed by release of mitochondrial cytochrome c into the cytosol and activation of caspase-mediated apoptosis. In contrast, proteasome inhibition did not induce any apparent changes in Bcl-2 protein levels. In addition, treatment with a proteasome inhibitor increased levels of ubiquitinated forms of Bax protein, without any effects on Bax mRNA expression. We also established a cell-free Bax degradation assay in which an *in vitro*-translated, [<sup>35</sup>S]-labeled Bax protein can be degraded by a tumor cell protein extract, inhibitable by addition of a proteasome inhibitor or depletion of the proteasome or ATP. The Bax degradation activity can be reconstituted by addition into the proteasome-depleted supernatant of a purified 20S proteasome or proteasome-enriched fraction. Finally, by using tissue samples of human prostate adenocarcinoma, we demonstrated that increased levels of Bax ubiquitination and degradation correlated well with decreased levels of Bax protein and increased Gleason Scores of prostate cancer. Our studies suggest that ubiquitin/proteasome-mediated Bax degradation is a novel survival mechanism in human cancer cells and that selective targeting this pathway should provide a unique approach for prevention and treatment of human cancers.

**(-)-EGCG or peptidyl proteasome inhibitor LLL induces apoptosis in prostate cancer PC-3 and LNCaP, but not DU145, cells in association with Bax accumulation.** Because Bax is an apoptosis inducer (4-6) and Bax expression is increased to a high level by EGCG in PC-3 and LNCaP, but not DU-145, cells (Figs. 3, 4), we hypothesized that EGCG could induce apoptosis in PC-3 and LNCaP, but not DU-145, cells. Indeed, in a concentration-dependent experiment, (-)-EGCG activated caspase-9 and caspase-3 activities only in PC-3, but not DU145 cells (Fig. 5A and B). Furthermore, in a kinetics experiment, (-)-EGCG activated caspase-3 activity for up to 7.5-fold in LNCaP cells, but only ~2-fold in DU145 cells (Fig. 5D). Consistent with that finding, (-)-EGCG induced apoptosis-specific PARP cleavage only in LNCaP, but not DU145 cells (Fig. 5C).

In addition, we found that DU145 cells were more resistant to the proteasome inhibitor LLL-induced apoptosis than both LNCaP and PC-3 cells. After LLL treatment, 50% of PARP was cleaved in PC-3 or LNCaP cells between 12-24 h, whereas only <10% PARP cleaved in DU145 cells at 24 h (Fig. 6).

**Dietary Tannic Acid Potently Inhibits Tumor Cell Proteasome Activity, Increases p27 and Bax Expression, and Induces G<sub>1</sub> Arrest and Apoptosis** (27 and Appendix). Previous animal studies have suggested that a dietary polyphenol known as tannic acid (TA) exhibits anticarcinogenic activity in chemically induced cancers although the involved molecular target remains unknown. In addition, proteasome inhibitors

have been shown to be able to suppress human tumor growth in nude mice. Most recently we have reported that ester-bond containing tea polyphenols are potent proteasome inhibitors *in vitro* and *in vivo*. We have hypothesized that TA which contains multiple similar gallate moieties linked by ester bonds should inhibit the proteasome activity. Here, we report that indeed TA potently and specifically inhibits the chymotrypsin-like activity of purified 20S proteasome ( $IC_{50} = 0.06 \mu\text{g/ml}$ ), 26S proteasome of Jurkat T cell extracts, and 26S proteasome of living Jurkat cells. Inhibition of the proteasome by TA in Jurkat cells results in accumulation of two natural proteasome substrates, the cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> and the pro-apoptotic protein Bax, followed by growth arrest in G<sub>1</sub> and apoptosis induction. Our present study suggests that TA targets and inhibits the proteasome in tumor cells, which may contribute to the previously observed anticarcinogenic activity of TA.

### Lung Cancer System

In this part of the report, we have presented results generated mainly from human lung cancer cells. We have used tea polyphenols and peptidyl proteasome inhibitors. In addition to Bax as a potential mediator of proteasome inhibition, we have also investigated whether decreased Bcl-2 expression contributes to the induced apoptosis.

**G<sub>1</sub> phase-dependent expression of Bcl-2 protein in human tumor cells** (28 and see Appendix). We hypothesized that (i) some apoptosis regulators are periodically expressed during the cell cycle progression and (ii) if so, cellular response to chemotherapeutic agents should be cell cycle-dependent. To test the first hypothesis, human Jurkat T cells were synchronized at the G<sub>1</sub>/S boundary by aphidicolin, followed by removal of the drug and further incubation of the cells in fresh growth medium until they progressed into the G<sub>1</sub> phase of the next cycle. At each time point, cells were harvested for measurement of the cell cycle distribution (by flow cytometry) and Bcl-2-like protein expression (Western blot assay) (Fig. 7).

Treatment with aphidicolin for 24 h accumulated 69% of the Jurkat cells in G<sub>1</sub> and 30% in early S phase (Fig. 7A, 0 h). Immunoblot with a combination of Bcl-2 and Bax antibodies revealed a high level of Bcl-2 and a low level of Bax protein in these cells (Fig. 7B, lane 1). At 6 h after release from the aphidicolin block, the G<sub>1</sub> population was decreased by 37%, associated with 25% increase in S and 12% increase in G<sub>2</sub>/M population (Fig. 7A). At this time, the Bcl-2 protein level was significantly decreased while Bax level remained unchanged (Fig. 7, B, C, lanes 2 vs. 1), resulting in a decrease in the Bcl-2/Bax ratio. At 10 h after removal of aphidicolin, both G<sub>1</sub> and S populations were decreased, associated with a marked increase in G<sub>2</sub>/M cell population (Fig. 7A). This was accompanied by a further decrease in the level of Bcl-2 protein (Fig. 7B, lane 3), indicating that Bcl-2 expression is not G<sub>2</sub>/M phase-dependent. At this time, the level of Bcl-2 became even lower than that of Bax that remained unchanged (Fig. 7, B, C, lanes 3 vs. 2). At 18 h, 70% of these cells had reentered into G<sub>1</sub> and 27% even into early S phase (Fig. 7, A), accompanied by reappearance of highly expressed Bcl-2 protein and unchanged expression of Bax protein (Fig. 7, B, C, lanes 4 vs. 3). In contrast to the dramatic changes in expression of Bcl-2 during the cell cycle progression, levels of the anti-apoptotic Bcl-X<sub>L</sub>, the pro-apoptotic Bcl-X<sub>S</sub> and the pro-apoptotic Bak proteins changed only slightly during this process (Fig. 7, D-F). Therefore, Bcl-2 is the only member of the family we examined that demonstrated a significant cell cycle-dependent protein expression in Jurkat T cells.

To further study the G<sub>1</sub> phase-dependent expression of Bcl-2 protein, we synchronized, by either serum starvation or aphidicolin treatment, several other human tumor cell lines, as well as human normal lung WI-38 and SV40-transformed WI-38 (VA-13) cell lines, and measured Bcl-2 protein levels in different phases of the

cell cycle. Again, expression of Bcl-2 protein was found to be the highest in G<sub>1</sub> phase of the cell cycle under all the tested experimental conditions (28; see the enclosed Mol Pharm. reprint).

**G<sub>1</sub> phase-dependent expression of Bcl-2 mRNA and protein correlates with chemo-resistance of human cancer cells (28).** Similar to the changes in Bcl-2 protein levels, its mRNA expression was also G<sub>1</sub> phase-specific, whereas the level of a Bcl-2 cleavage activity remained constitutive. We then tested the second hypothesis. When treated with an anticancer drug (etoposide or cisplatin) or the kinase inhibitor staurosporin, the cells containing a high G<sub>1</sub> population and a high Bcl-2 protein level were much more resistant to the induced apoptosis than the cells containing a high S phase population and a low Bcl-2 protein level. Constitutive overexpression of Bcl-2 protein in Jurkat T cells completely blocked the S phase-associated sensitivity to these apoptosis stimuli. It appears that the cell cycle-dependent Bcl-2 protein expression contributes to the regulation of chemo-sensitivity and apoptotic commitment of human tumor cells (28).

**G<sub>1</sub> phase-dependent expression of Bcl-2 protein in mitochondria.** We found that when cytosolic (C) and mitochondrial membrane-bound (M) fractions were isolated from exponentially growing Jurkat T cells, almost all the Bcl-2 protein was detected in the mitochondrial fraction (Fig. 8B). In comparison, most of Bax was found in the cytosolic fraction although a portion of Bax was detected in the mitochondrial fraction (Fig. 8A). As a control, expression of mitochondrial cytochrome oxidase (COX; 26, 29, 30) was only detected in the membrane fraction (Fig. 8C). To study localization of Bcl-2 protein during the cell cycle, Jurkat T cells were synchronized at G<sub>1</sub>/S by aphidicolin, followed by release. At each time point, aliquots of cells were used for flow cytometry (see Fig. 7A) and cytosolic/mitochondrial fractionation, which were subsequently used for determination of Bcl-2 protein expression (Fig. 9). The aphidicolin-synchronized cells (0 h) contained a high G<sub>1</sub> population (~70%; Fig. 7A) and expressed no cytosolic Bcl-2 protein but high mitochondrial Bcl-2 protein (Fig. 9A, lanes 1 vs. 4; compare to Fig. 2). At 6 h after release, ~40% of the G<sub>1</sub> cells entered into S/G<sub>2</sub>/M (Fig. 7A), associated with a significant decrease in the mitochondrial Bcl-2 level (Fig. 9A, lanes 5 vs. 4; compare to Fig. 7B). When most of the cells (70%) reentered into the G<sub>1</sub> phase of the next cycle (18 h; Fig. 7A), the mitochondrial Bcl-2 level was increased again (Fig. 9A, lane 6; compare to Fig. 7B). As a control, the mitochondrial COX level was not decreased, instead slightly increased, during this process (Fig. 9B, lanes 4-6). This result suggests that G<sub>1</sub> tumor cells express high levels of Bcl-2 protein in mitochondria, associated with resistance of these cells to apoptosis induction (28), and that S phase tumor cells express low mitochondrial Bcl-2 protein, correlated with increased chemo-sensitivity (28). It appears that down-regulation of the mitochondrial Bcl-2 expression in G<sub>1</sub> or non-growing tumor cells is an important strategy for improving current cancer therapies.

**Green tea treatment of G<sub>1</sub> lung transformed and tumor cells abolishes Bcl-2 protein expression and significantly enhances VP-16-induced apoptosis.** We also reported that growth-arrest of transformed lung cells (VA-13) accumulated high levels of Bcl-2 protein (28). Indeed, serum starvation for 96 h accumulated 84% of VA-13 cells in G<sub>0</sub>/G<sub>1</sub> phase (Fig. 10B, *a*), which expressed high levels of Bcl-2 protein (Fig. 10A, lane 1). We then examined whether green tea extract (GTE) could decrease the Bcl-2 protein expression in the G<sub>1</sub>-arrested transformed lung cells. Incubation of the growth-arrested VA-13 cells with fresh serum-free medium containing GTE (50 or 100 µg/ml) for 3 or 6 h completely abolished expression of Bcl-2 protein (Fig. 10A, lanes 3, 5, 11 vs. 1). Such a tea treatment had little effect on the cell cycle distribution and only slightly increased apoptotic population (by ~5%; Fig. 10B, *c-e* vs. *a*). Incubation of the G<sub>1</sub> VA-13 cells with the control vehicle (H<sub>2</sub>O) neither affected high Bcl-2 expression (Fig. 10A, lanes 2, 4, 10 vs. 1), the cell cycle distribution (Fig. 10B, *b* vs. *a*), nor induced apoptosis (Fig. 10B, *b*). To study whether tea pretreatment increases cellular chemo-sensitivity, the G<sub>1</sub> VA-13 cells pretreated with either tea or H<sub>2</sub>O were incubated with 20 µM VP-16 for 24 h. The VP-16 treatment had little effects on levels of Bcl-2 protein expression in all the pretreated cells (Fig. 10A, lanes 6-9 vs. 2-5). When the H<sub>2</sub>O-pretreated G<sub>1</sub> VA-13 cells were treated with VP-16, apoptotic population was increased only by <5% (Fig. 10B, *f, g* vs. *b*), supporting the idea that non-proliferating lung

tumor or transformed cells are resistant to chemotherapeutic agents (31-33). However, VP-16 treatment of the tea-pretreated cells significantly induced apoptosis (by 21-27%; Fig. 10B, *h-j*). The levels of VP-16-induced apoptosis were tea concentration-dependent: higher apoptotic population was observed in the cells pretreated with tea at 100  $\mu\text{g/ml}$  than at 50  $\mu\text{g/ml}$  (Fig. 10B, *j* vs. *h*).

We then performed a similar experiment with human lung adenocarcinoma A-549 cells. Basal levels of Bcl-2 protein were high in asynchronous A549 cells (Fig. 11A, lane 1), consistent with previous reports (34, 35). Incubation of these cells with 50  $\mu\text{g/ml}$  GTE decreased Bcl-2 protein expression in a time-dependent manner (Fig. 11A, lanes 2-4 vs. 1). Serum starvation for 96 h accumulated most of A549 cells in G<sub>1</sub> phase (Fig. 11E, panel *a*), that expressed high levels of Bcl-2 protein (Fig. 11B, lane 1). Treatment of these G<sub>1</sub> A549 cells with serum-free medium containing 10  $\mu\text{g/ml}$  GTE, but not H<sub>2</sub>O, for 3 to 6 h blocked Bcl-2 expression (Fig. 11B, lanes 2-5 vs. 1). Such a GTE (Fig. 11E, *b*) or H<sub>2</sub>O (similar to Fig. 11E, *c*) treatment for 6 h induced little cell death.

However, VP-16 post-treatment of the G<sub>1</sub> A549 cells pretreated with GTE but not H<sub>2</sub>O induced significant apoptosis. Loss of pro-caspase-3/p32, which represents its activation (7-9), was observed in G<sub>1</sub> A549 cells pretreated for 6 h with 10  $\mu\text{g/ml}$  GTE and then post-treated with 20  $\mu\text{M}$  VP-16 for 6 or 20 h (Fig. 11C, lanes 3, 4 vs. 2, 1). In contrast, high levels of pro-caspase-3 were found in cells pretreated with H<sub>2</sub>O and post-treated with VP-16 (Fig. 11C, lanes 6, 7 vs. 5, 1). Similarly, when exposed to 20  $\mu\text{M}$  VP-16 for 24 h, cleavage of PARP was detected only in G<sub>1</sub> A549 cells pretreated with 10  $\mu\text{g/ml}$  GTE but not H<sub>2</sub>O (Fig. 11D, lanes 2, 3 vs. 1). Finally, VP-16 post-treatment for 24 h of GTE-, but not H<sub>2</sub>O-, pretreated G<sub>1</sub> A549 cells increased apoptotic pre-G<sub>1</sub> population (Fig. 11E, panels *d* vs. *c* vs. *a*). The used concentrations of GTE (10-50  $\mu\text{g/ml}$ ) in the above experiments contain tea polyphenols in an  $\mu\text{M}$  range (as calculated by the percentage and the mass of each polyphenol), which is close to the physiological concentrations of tea polyphenols in the serum of tea drinkers (36, 37). We conclude that tea at physiological concentrations can down-regulate Bcl-2 protein expression and consequently enhance chemo-sensitivity in otherwise drug-resistant human lung cancer and transformed cells.

**Resistance of normal WI-38 cells to release cytochrome c upon proteasome inhibitor treatment.** Previously we reported that tumor and SV40-transformed cells were much more sensitive to proteasome inhibitor-induced apoptosis than normal WI-38 cells (38) and that tumor cell death induced by proteasome inhibitors was associated with cytochrome c release and dependent on caspase activation (26). We hypothesized that resistance of normal human cells to proteasome inhibitor-induced apoptosis is due to failure or resistance of these cells to release mitochondrial cytochrome c into the cytosol. To test this hypothesis, human normal WI-38 and SV-40-transformed WI-38 (VA-13) cells were treated with the tripeptidyl proteasome inhibitor LLnL for up to 24 h, followed by measurement of apoptosis-specific PARP cleavage and cytosolic cytochrome c levels.

About 30% of PARP protein was cleaved in VA-13 cells treated with LLnL for 8 h, and almost all the PARP protein was cleaved by 24 h (Fig. 12A, lanes 1-4). In contrast, when WI-38 cells were treated under the identical conditions, no PARP cleavage was detected at 8 h, and even after 24 h, only very low levels of the PARP cleavage fragment (PARP/p85) were detected (Fig. 12A, lanes 5-8). To measure release of mitochondrial cytochrome c into the cytosol in both cell lines, cytosolic fractions were prepared and used for Western blot assay with a specific cytochrome c antibody. Levels of cytosolic cytochrome c were significantly increased in LLnL-treated VA-13 cells, which started at 8 h (Fig. 12B, lanes 1-6). Levels of cytosolic cytochrome c were low in untreated WI-38 cells (Fig. 12B, lane 7), remained low after LLnL treatment for up to 16 h, and slightly increased only after 24 h-treatment (Fig. 12B, lanes 8-12). The observed different kinetics of cytochrome c release in both VA-13 and WI-38 cell lines were not due to unevenly loaded samples since the same cytochrome c antibody also detected a ~40 kDa protein band with unknown nature in these cytosolic fractions whose levels were relatively unchanged (Fig. 12B). Importantly, the kinetics of cytochrome c release

in both VA-13 and WI-38 cells matched well that of PARP cleavage (Fig. 12B vs. A). Therefore, a proteasome inhibitor-inducible pathway that controls cytochrome c release is suppressed in normal WI-38 cells.

**Resistance of normal WI-38 cells to accumulate mitochondrial Bax protein after proteasome inhibitor treatment.** Our previous studies suggested that before or associated with cytochrome c release in Jurkat T cells treated with a proteasome inhibitor, Bax was accumulated to mitochondria (26). We then tested the idea whether resistance of WI-38 cells to release of cytochrome c by proteasome inhibition (Fig. 12B) was associated with failure of these cells to accumulate Bax protein in mitochondria. We first measured Bax protein levels using whole cell extracts prepared from VA-13 and WI-38 lines. LLnL treatment of VA-13 cells increased levels of Bax and a Bax-related, 55 kDa polypeptide (Fig. 13A). By 24 h, levels of both Bax and p55 were increased by 3- to 4-fold (Fig. 13A). A similar p55 was found in both Jurkat T cells treated with a proteasome inhibitor and high-grade prostate cancer tumor samples, which can be detected by antibodies to both Bax and ubiquitin proteins (26). These data suggest that p55 may contain ubiquitinated Bax. In contrast to VA-13 cells, Bax protein levels in WI-38 cells were not increased up to 16 h-treatment with LLnL, and only slightly increased (< 2-fold) after 24 h-treatment (Fig. 13B). A similar p55 was also detected in WI-38 cells, whose levels were slightly increased by LLnL treatment for 24 h, but not shorter (Fig. 13B). An extra band of ~35 kDa with unknown nature was found only in WI-38, but not in VA-13, cells (Fig. 13B vs. A). The basal levels of Bcl-2 protein were lower in WI-38 than VA-13 cells (Fig. 13C, lanes 5 vs. 1). Bcl-2 protein levels were slightly increased in VA-13 cells, while decreased in WI-38 cells, after a 24 h-treatment with LLnL (Fig. 13C).

We then measured Bax protein levels in both cytosolic and mitochondrial fractions of VA-13 and WI-38 cells. Similar to Jurkat T cells (26), when the transformed VA-13 cells were treated with LLnL, cytosolic Bax levels were decreased, associated with increased levels of mitochondrial Bax protein (Fig. 14, lanes 4-6 vs. lanes 10-12). However, when WI-38 cells were analyzed under identical conditions, little or no mitochondrial Bax protein was detected although cytosolic Bax protein levels were decreased (Fig. 14, lanes 7-9 vs. 1-3). Therefore, WI-38 cells failed to accumulate Bax protein to mitochondria after proteasome inhibitor treatment, which was associated with resistance of these cells to release mitochondrial cytochrome c and induce apoptosis (Fig. 12).

**Inhibition of the proteasome activity induces apoptosis and inhibits the growth of the human lung adenocarcinoma A-549 in nude mice (39).** Treatment of A-549 cells with the dipeptidyl proteasome inhibitor CEP1612 results in accumulation of two proteasome natural substrates, the cyclin-dependent kinase inhibitors p27 and p21, consistent with its ability to inhibit proteasome activity in intact cells. Furthermore, CEP1612 induces apoptosis in A459 cells as evident by caspace-3 activation and PARP cleavage. Treatment of A-549 tumor bearing (s.c.) nude mice with CEP1612 (10 mg/kg per day, i.p. for 31 days) resulted in massive induction of apoptosis (as measured by TUNEL assay) and significant 68% tumor growth inhibition ( $p < 0.05$ ) (Fig. 9). Furthermore, immunostaining of tumor specimens also demonstrated *in vivo* accumulation of p27 and p21 following CEP1612 treatment. The results suggest that CEP1612 is a promising candidate for further development as an anticancer drug and demonstrate the feasibility of using proteasome inhibitors as novel anti-tumor agents.

### **(3) KEY RESEARCH ACCOMPLISHMENTS:**

- a. Bax is required for a peptidyl proteasome inhibitor-induced apoptosis in human prostate or lung cancer cells.
- b. Bax is required for tea polyphenol EGCG-induced apoptosis in human prostate or lung cancer cells.
- c. Resistance of human normal lung cells to proteasome inhibitor treatment is associated with failure of these cells to accumulate Bax to mitochondria.
- d. Decreased Bcl-2 expression in mitochondria sensitizes tumor cells to induction of apoptosis.

### **(4) REPORT OUTCOMES:**

- **Three manuscripts are published and one manuscript is in preparation (see Appendix).**
- **Three Abstracts are presented in the following meetings:**

Smith DM and Dou QP. Green tea targets human tumor cell DNA synthesis and consequently induces apoptosis. Poster presentation. New Molecular Targets for Cancer Therapy, St. Petersburg Beach, FL, October 14-17, 2000

Smith DM, Nam S and Dou QP. Studies on tumor related targets of green tea polyphenols. Poster presentation. 2<sup>nd</sup> International Conference. Mechanisms of Cell Death and Disease: Advances in Therapeutic Intervention, North Falmouth, MA, June 2-6, 2001

Kazi A, Smith DM, Zhong Q and Dou QP. Down Regulation of Bcl-X<sub>L</sub> Protein Expression by Green Tea Polyphenols Is Associated with Prostate Cancer Cell Apoptosis. Poster presentation. AACR-NCI-EORTC. Molecular Targets and Cancer therapeutics, Miami Beach, FL, October 29-November 2, 2001

- **One Ph.D. degree will be obtained (David M. Smith is expected to graduate in Spring 2002), which is partially supported by this Award.**
- **One grant has been awarded, based on the work supported by this award:**

“Tea Targeting Proteasome: A Role in Cancer Prevention” (Determine potency, selectivity and effects of EGCG and EGC to inhibit the proteasome, the Bax degradation, activity in prostate cancer cell extracts and cell cultures; **Principal Investigator: Q. Ping Dou, Ph.D.**)

Agency: NIH/NCI; Type: R03 (CA091282-01, Years 1-2); Period: April 1, 2001 to March 31, 2003.

- **Two grants have been submitted or resubmitted, based on the work supported by this award:**

American Institute for Cancer Research. Tea Polyphenols Target Proteasome-Mediated Bax Degradation Pathway: Significance in Prostate Cancer Prevention and Treatment. Principal Investigator: Q. Ping Dou (15%). 02/01/01-01/31/03. Total Direct Costs: \$150,000; Total Indirect Costs: \$15,000 (**invited for a resubmission**).

NIH R01. Synthetic EGCG Analogs and Prostate Cancer Prevention. Principal Investigator: Q. Ping Dou (25%). 07/01/02-06/30/07. Total Direct Costs: \$1,250,000; Total Indirect Costs: \$297,985

## **(5) CONCLUSIONS:**

- a. When prostate or lung cancer cells containing wild-type Bax protein (such as LNCaP and PC-3) are treated with a peptidyl or tea polyphenol proteasome inhibitor, Bax protein is accumulated, followed by induction of apoptosis. Prostate or lung cancer cells without Bax protein (such as DU145) are more resistant to the proteasome inhibitor-induced apoptosis than prostate or lung cancer cells containing wild-type Bax protein.
- b. Resistance of normal human lung WI-38 cells to accumulate mitochondrial Bax protein after proteasome inhibitor treatment is associated with failure of these cells to release cytochrome c and undergo apoptosis.

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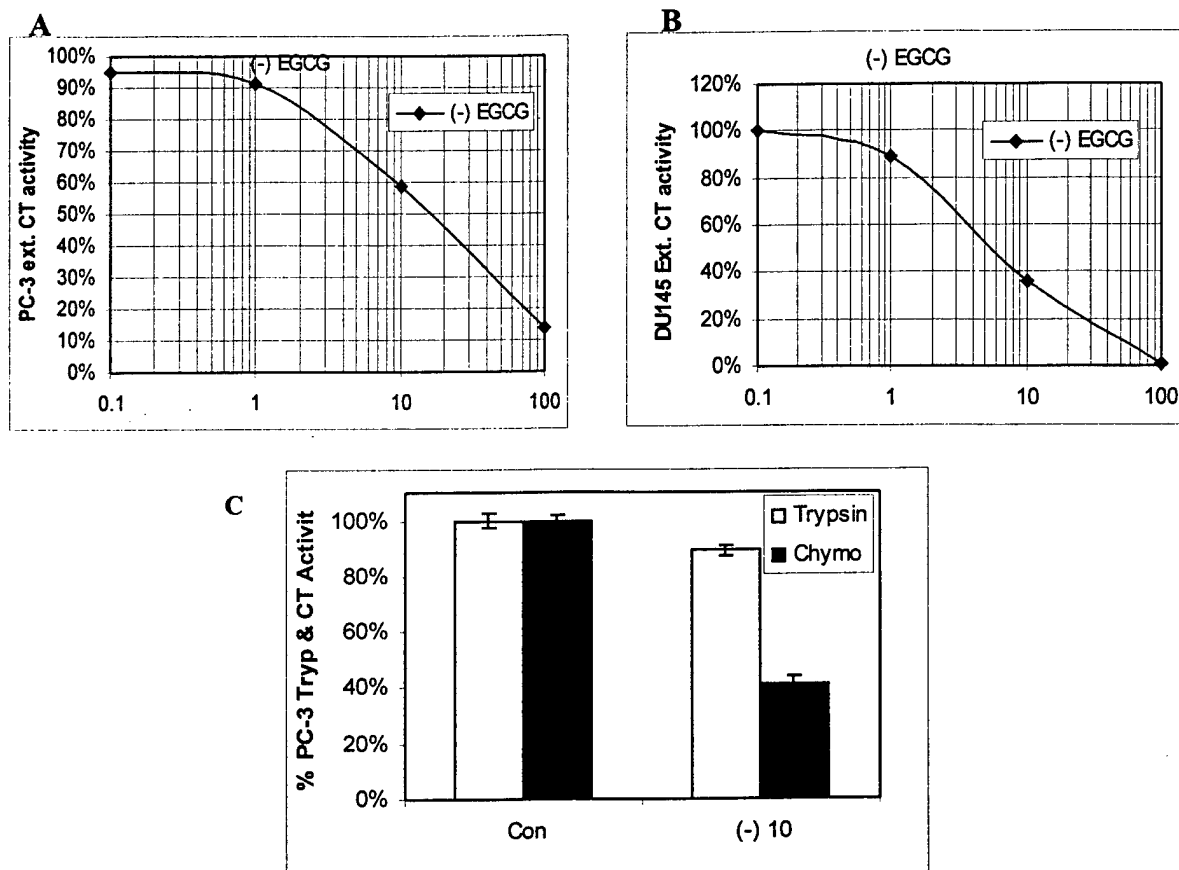


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**(7) APPENDICES:**

Four reprints, 14 figures and one copy of CV are enclosed.

**Fig. 1**



**Fig. 1.** Effects of EGCG on inhibiting the proteasomal chymotrypsin-like and trypsin-like activities in prostate cell extract. 20  $\mu$ g protein extract of PC-3 (A, C) or DU145 (B) was used for measurement of the proteasomal chymotrypsin- (A, B, C) or trypsin-like (C) activity, in the presence of (-)-EGCG at indicated concentrations ( $\mu$ M). In C, 10  $\mu$ M of (-)-EGCG was used. Con, PC-3 extract alone without an EGCG analog.

Fig. 2

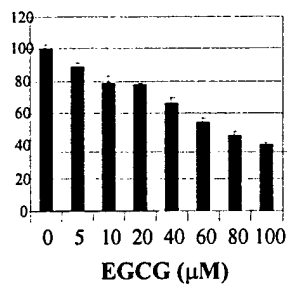


Fig. 3

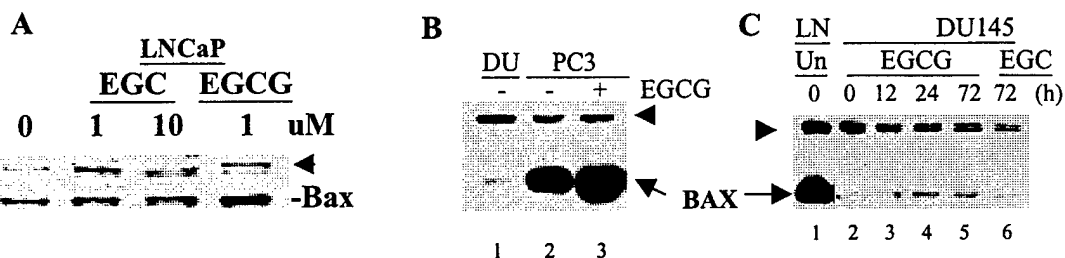


Fig. 4

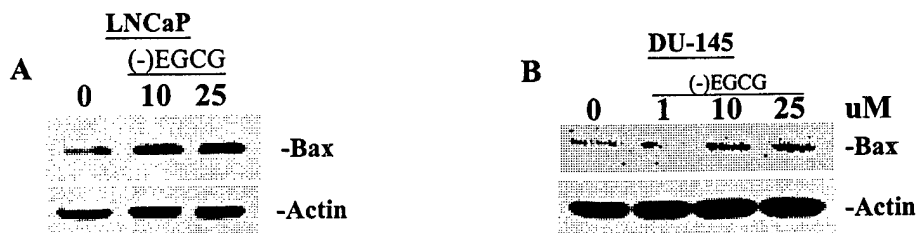
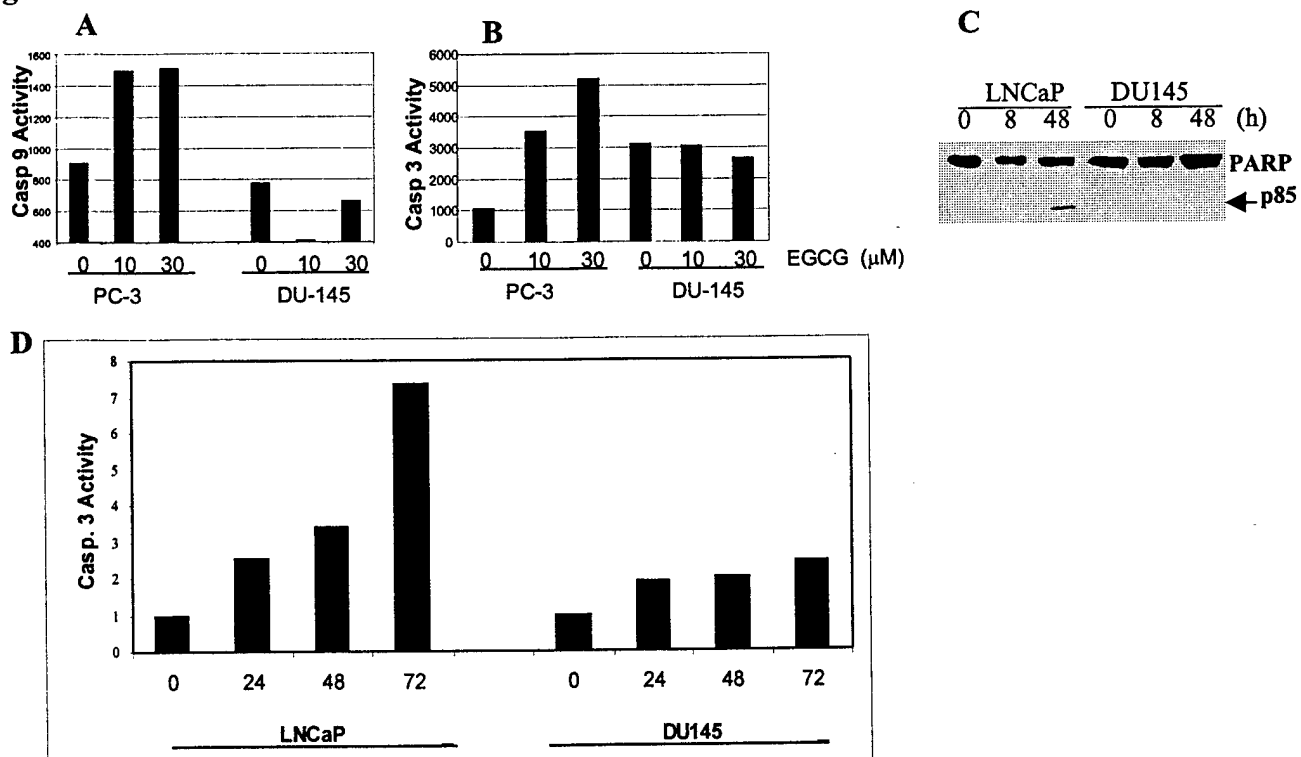


Fig. 5



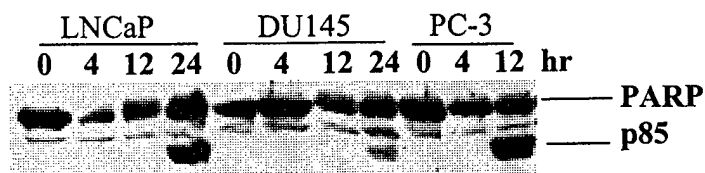
**Fig. 2.** Inhibition of the proteasome activity by (-)-EGCG in intact PC-3 cells. Human prostate cancer PC-3 cells were pre-incubated for 12 h with either the solvent (0  $\mu$ M) or (-)-EGCG at the indicated concentrations, followed by an additional 2 h-incubation with 20  $\mu$ M Suc-Leu-Leu-Val-Tyr-AMC (for the chymotrypsin-like activity of the proteasome). The medium was collected and the free AMC groups were measured (3).

**Fig. 3.** Effects of (-)-EGCG on Bax accumulation in prostate cancer cells. **(A)** Exponentially grown LNCaP cells (lane 1) were treated with 1  $\mu$ M (-)-EGCG (lane 4) or 1 or 10  $\mu$ M (-)-EGC (lanes 2, 3) for up to 12 h. **(B)** Growing PC-3 cells (lane 2) were treated with 10  $\mu$ M (-)-EGCG for 24 h (lane 3). Exponentially grown DU145 cells were shown in lane 1. **(C)** Growing DU145 cells (lane 2) were treated with either 50  $\mu$ M (-)-EGCG for up to 72 h (lanes 3-5) or 50  $\mu$ M (-)-EGC for 72 h (lane 6). Growing LNCaP cells (LN, untreated) were shown in lane 1. After each treatment, Western blotting was performed with an anti-Bax antibody. Bax (21 kD) is indicated by an arrow. A high-molecular-weight band, recognized by the anti-Bax antibody, is indicated by an arrowhead. Although its nature remains unknown, this high-molecular-weight band can be used as a loading control.

**Fig. 4.** Potencies of (-)-EGCG on Bax accumulation in prostate cancer cells. **(A)** Exponentially grown LNCaP cells (lane 1) were treated for 12 h with (-)-EGCG at 10 or 25  $\mu$ M. **(B)** Exponentially grown DU145 cells (lane 1) were treated for 48 h with (-)-EGCG at 1, 10 or 25  $\mu$ M. After each treatment, Bax and actin (as a loading control) protein levels were measured in Western blot assay.

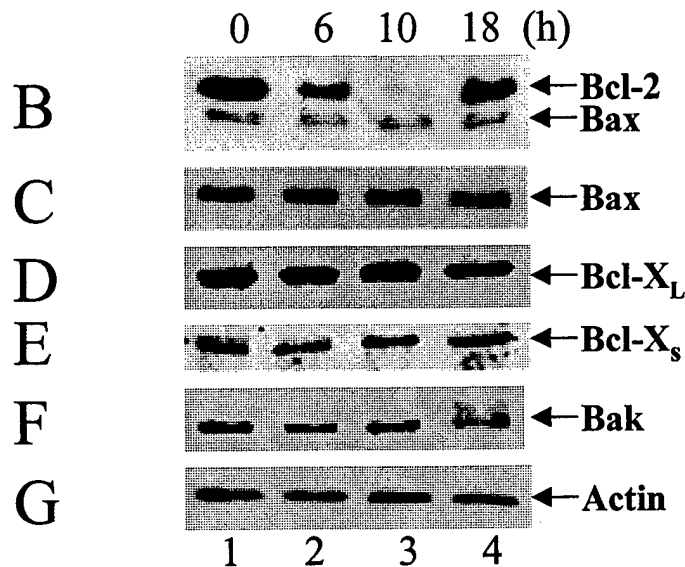
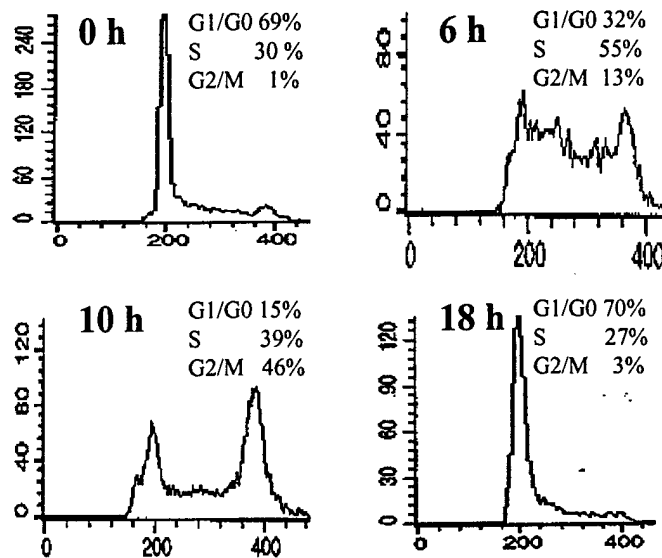
**Fig. 5.** Effects of (-)-EGCG on apoptosis induction in prostate cancer cells. **(A, B)** Growing PC-3 (bar 1) or DU145 (bar 4) cells were treated with 10 or 30  $\mu$ M (-)-EGCG for 48 h, followed by measuring cell-free caspase-9 and -3 activities. Briefly, protein extract (20  $\mu$ g) was incubated at 37 $^{\circ}$  C for 2 h in a buffer containing 50 mM Tris/pH 8.0 and a specific substrate for caspase-9 (Ac-LEHD-AFC) or -3 (Ac-DEVD-AMC; *Calbiochem*) at 20  $\mu$ M in a 96 well plate. After incubation, the liberated florescent AMC or AFC groups were measured by a Wallac Victor<sup>2</sup> 1420 Multilabel counter with 355/460 nM and 405/535 nM filters, respectively. **(C)** Growing LNCaP (lane 1) or DU145 (lane 4) cells were treated with 50  $\mu$ M (-)-EGCG for 8 or 48 h as indicated. After each treatment, apoptosis-specific PARP cleavage was measured. PARP and the p85/PARP fragment are indicated. **(D)** Growing LNCaP (0 h) or DU145 (0 h) cells were treated with 10  $\mu$ M (-)-EGCG for 24, 48, or 72 h as indicated. After each treatment, cell-free caspase-3 activity was measured as described above.

**Fig. 6.**



**Fig. 6.** Effects of LLL on apoptosis induction in prostate cancer cells. LNCaP, DU145 and PC-3 cells (0 h) were treated with 50  $\mu$ M of LLL, followed by measurement of apoptosis-specific PARP cleavage.

Fig. 7 A



**Fig. 7.** Expression of Bcl-2 family members in human Jurkat T cells during the cell-cycle progression. Human Jurkat T cells were treated with aphidicolin at 3  $\mu$ g/ml for 24 h (0 h), followed by release for up to 18 h (see **Materials and Methods**). At each time point, one-third of the cells were harvested for flow cytometry analysis of the cell cycle (A), one-third were for whole cell extraction and Western blot assay (B-G), and one-third were incubated with VP-16, followed by measurement of apoptosis (see Fig. 7 in the reprint). In panel B, the filter was first incubated with a specific antibody to Bcl-2, rinsed, and then incubated with a Bax antibody. Panels C was from the anti-Bax antibody alone. Panels D to G were results using specific antibodies to Bcl-X<sub>L</sub>, Bcl-X<sub>S</sub>, Bak and Actin, respectively.

Fig. 8.

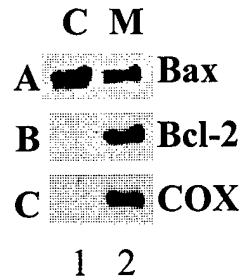


Fig. 9.

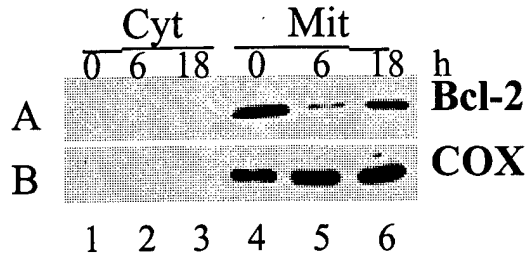
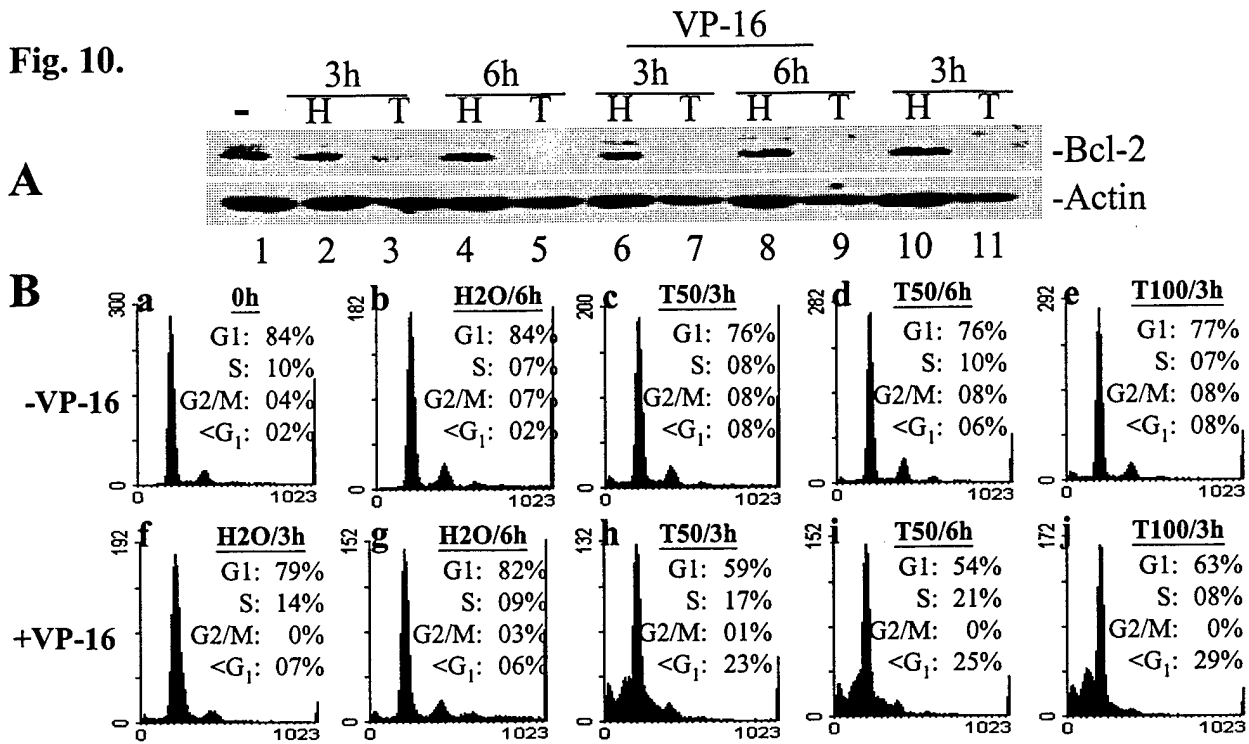


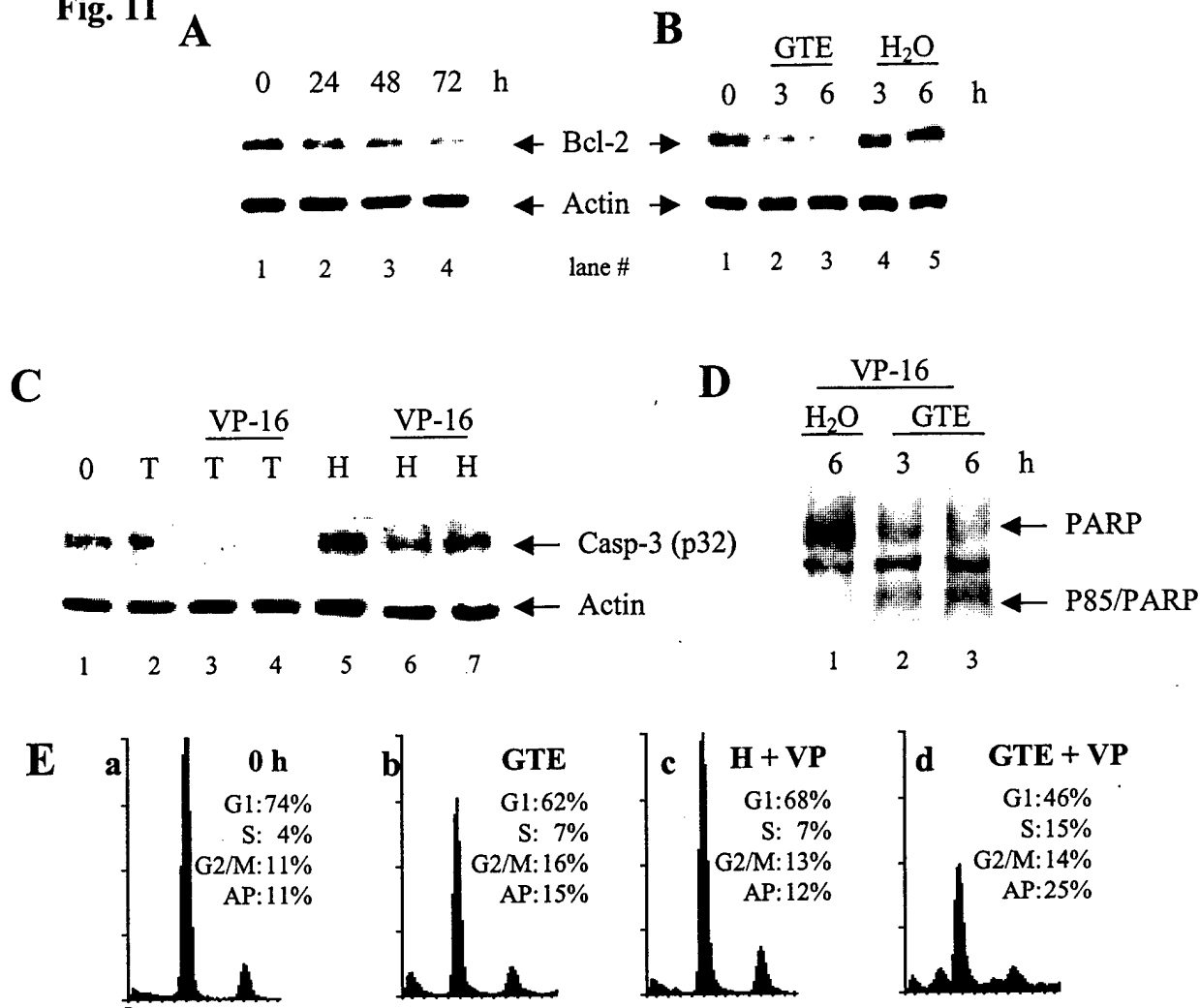
Fig. 10.



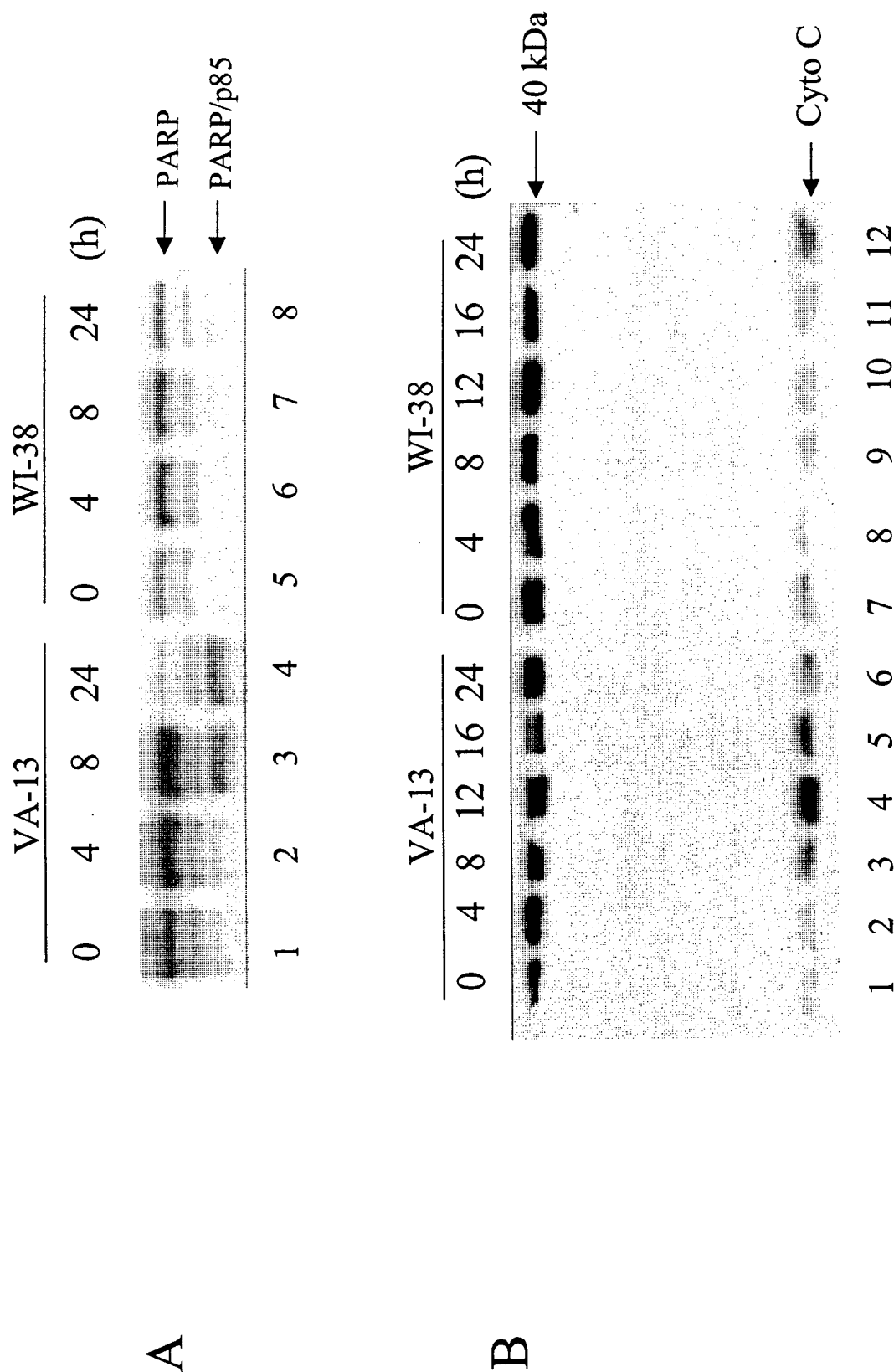
**FIG. 8.** Mitochondrial localization of Bcl-2. Cytosolic (C) and membrane-bound (M, mainly containing mitochondria) fractions were prepared from asynchronous Jurkat T cells, followed by Western blot with antibodies to Bax, Bcl-2 and COX.

**FIG. 9.** Cell cycle-dependent mitochondrial Bcl-2 expression. Jurkat T cells were treated with aphidicolin at 3  $\mu$ g/ml for 24 h (0 h), followed by release for indicated hrs. At each time point, cells were prepared for flow cytometry (see Fig 1A) and cytosolic (Cyt)/ mitochondrial (Mit) fractions, which were subsequently immunoblotted first with anti-Bcl-2 antibody (A), and then reblotted with anti-cytochrome oxidase subunit II (COX; B).

**FIG. 10.** Transformed lung cell line VA-13 was serum-starved for 96 h (0h or -). Aliquots of the cells were then treated for 3 or 6 h with either a green tea extract at 50 (in A, indicated by T, lanes 3, 5, 7, 9; in B, indicated by T50) or 100  $\mu$ g/ml (in A, indicated by T, lane 11; in B, indicated by T100) or the control solvent H<sub>2</sub>O (in A, indicated by H, lanes 2, 4, 6, 8, 10; in B, indicated by H<sub>2</sub>O). After that, some aliquots of the cells were post-treated with 20  $\mu$ M VP-16 for 24 h (A, lanes 6-9; B, indicated by +VP-16). Samples were used for both Western blotting (A) and flow cytometry (B). Actin levels were measured as a loading control in A.

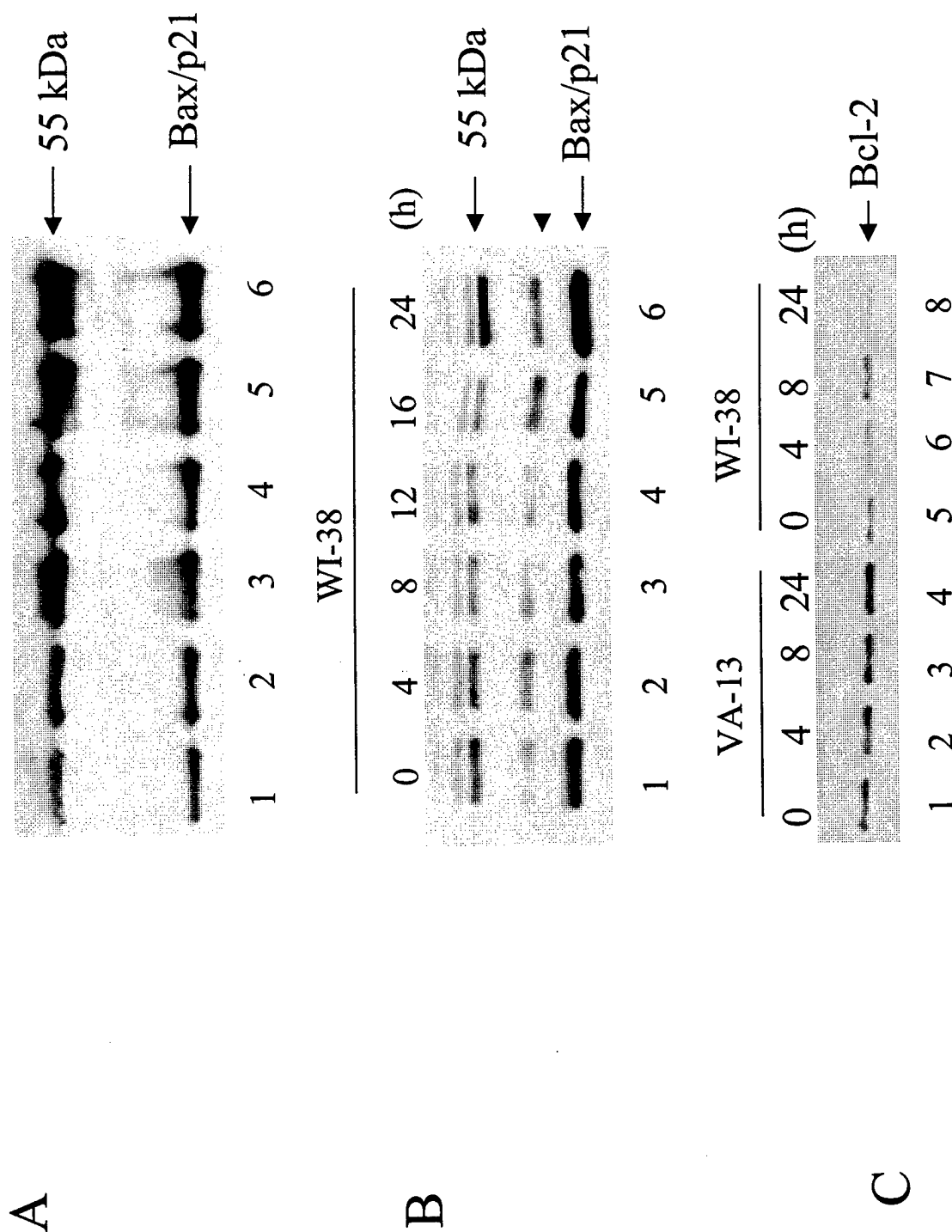
**Fig. 11**

**FIG. 11.** (A) Asynchronous human lung-cancer A549 cells (lane 1) were treated with 50  $\mu\text{g/ml}$  GTE for 24, 48 or 72 h (lanes 2-4). (B-E) A549 cells were serum-starved for 96 h (indicated by 0 h), followed by a pre-treatment with 10  $\mu\text{g/ml}$  GTE or the control vehicle H<sub>2</sub>O. Aliquots of the pretreated A549 cells were then post-treated with 20  $\mu\text{M}$  VP-16. (B) Bcl-2 levels were decreased in whole protein extracts of A549 cells pretreated for 3 or 6 h with GTE but not H<sub>2</sub>O. Actin levels were measured as a control. (C) Loss of pro-caspase-3/p32, which represents its activation, was observed in G<sub>1</sub> A549 cells pretreated for 6 h with GTE (indicated by T) and then post-treated with VP-16 for 6 (lane 3) or 20 h (lane 4). In contrast, high levels of pro-caspase-3 were found in cells pretreated for 6 h with H<sub>2</sub>O (indicated by H) and post-treated with VP-16 for 6 (lane 6) or 20 h (lane 7). (D) A 24 h post-treatment with VP-16 induced PARP cleavage in GTE- (but not H<sub>2</sub>O-) pretreated (for 3 or 6 h) G<sub>1</sub> A549 cells. (E) A 24 h post-treatment with VP-16 induced apoptotic pre-G<sub>1</sub> population in G<sub>1</sub> A549 cells pretreated for 6 h with GTE but not H<sub>2</sub>O (H). 0 h, serum-starved A549 cells.

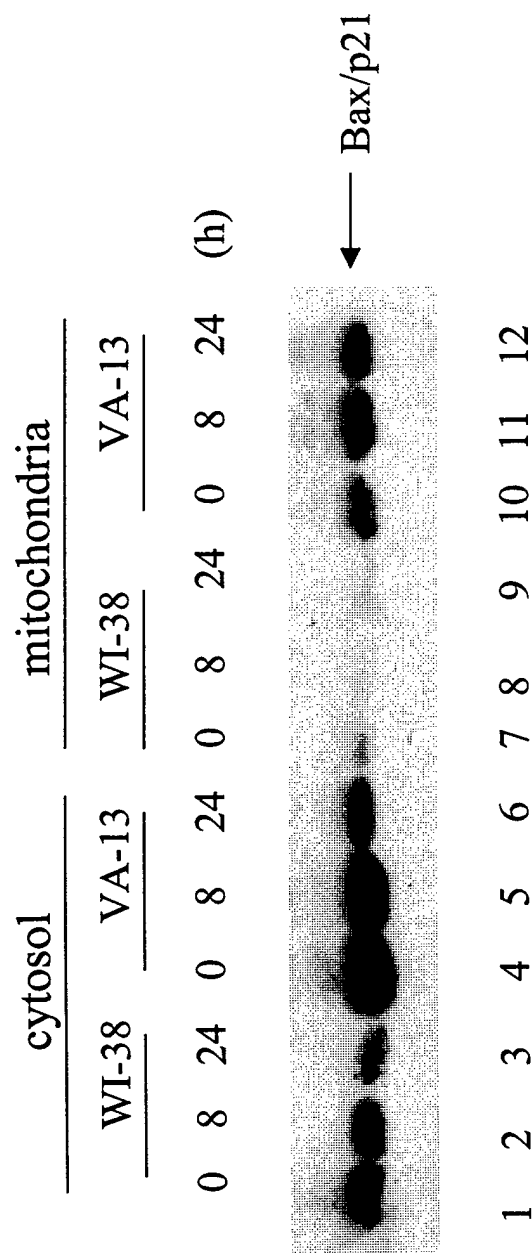


**Fig. 12.** Both WI-38 and VA-13 cell lines were treated with proteasome inhibitor LLnL (50 uM) for up to 24 h, followed by measurement of PARP cleavage (A) and cytochrome c release (B). (PI: Q. Ping Dou)





**Fig. 13.** Levels of Bax (A, B) and Bcl-2 (C) proteins were measured in the experiment described in fig. 12. (PI; Q. Ping Dou)



**Fig. 14.** Both WI-38 and VA-13 cell lines were treated with proteasome inhibitor LLnL (50 uM) for up to 24 h, followed by isolation of mitochondrial and cytosolic fractions. The isolated fractions were used for Western Blot assay using a specific Bax antibody.

PI: Q. Ping Dou

# G<sub>1</sub> Phase-Dependent Expression of Bcl-2 mRNA and Protein Correlates with Chemoresistance of Human Cancer Cells

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## ABSTRACT

Recent experiments suggest an interconnection between cell proliferation and programmed cell death (apoptosis), although the detailed molecular mechanisms remain unclear. We have hypothesized that expression of some apoptosis regulators is cell cycle-dependent, which in turn influences tumor cell chemosensitivity in a cell cycle-dependent fashion. To test these hypotheses, we synchronized human leukemia Jurkat T, Neo (using aphidicolin), breast cancer MCF-7, normal fibroblast, and simian virus 40-transformed cells (by aphidicolin or serum starvation), and measured levels of several Bcl-2 family proteins. The highest expression of Bcl-2 protein was found in the G<sub>1</sub> phase of all the five cell lines tested. In contrast, levels of Bax protein remained relatively unchanged in four of the cell lines, and levels of Bcl-X<sub>L</sub>, Bcl-X<sub>S</sub>, and Bak proteins showed

little or no cell cycle-dependent changes in Jurkat T cells. Similar to the changes in Bcl-2 protein levels, its mRNA expression was also G<sub>1</sub> phase-specific, whereas the level of a Bcl-2 cleavage activity remained constitutive. When treated with an anticancer drug (etoposide or cisplatin) or the kinase inhibitor staurosporin, the cells containing a high G<sub>1</sub> population and a high Bcl-2 protein level were much more resistant to the induced apoptosis than the cells containing a high S phase population and a low Bcl-2 protein level. Constitutive overexpression of Bcl-2 protein in Jurkat T cells completely blocked the S phase-associated sensitivity to these apoptosis stimuli. The cell cycle-dependent Bcl-2 protein expression seems to contribute to the regulation of chemosensitivity and apoptotic commitment of human tumor cells.

Cell proliferation is tightly controlled in normal mammalian cells, but deranged in cancer cells (Pardee et al., 1978). The growth factor-mediated signals that drive the cell cycle progression and thereby cell proliferation have been linked to functions of several cell cycle-dependent regulators (Sherr and Roberts, 1999). Programmed cell death (apoptosis) is the process by which a cell will actively commit suicide under tightly controlled circumstances (Wyllie et al., 1980). Apoptosis occurs in two physiological stages, commitment and execution (Earnshaw, 1995; Martin and Green, 1995). It has been proposed that Bcl-2 family proteins are involved in the apoptotic commitment in mammalian cells (Green and Reed, 1998). Most recent experiments have demonstrated that several Bcl-2 family proteins are located in the outer mitochondrial membrane, where they control release of some caspase-activating proteins (such as cytochrome *c*) into the cytosol. Release of cytochrome *c* can be induced by proapoptotic Bcl-2 family proteins (such as Bax), but inhibited by antiapoptotic

Bcl-2 family proteins (such as Bcl-2). The ratio of proapoptotic Bcl-2 family members (such as Bax) to antiapoptotic members (such as Bcl-2), therefore, determines whether a cell is committed to apoptotic death or not (Green and Reed, 1998). Apoptotic execution in mammalian cells is initiated by activation of specific caspase proteases (Earnshaw, 1995; Martin and Green, 1995), which cleave important cellular target proteins, including poly(ADP-ribose) polymerase (PARP) (Lazebnik et al., 1994) and retinoblastoma protein (An and Dou, 1996), resulting in disassembly of the cell.

Homeostasis of cell numbers is achieved by balancing cell proliferation and cell death, suggesting an accurate coordination between these two processes. Indeed, recent experiments have shown that signal transduction pathways controlling cell proliferation and cell cycle progression are also involved in mediating apoptosis and that dysregulation of cell cycle progression is an important event for the initiation of apoptosis (Lee et al., 1993; Dou et al., 1995; Linette et al., 1996; Dou, 1997). Furthermore, expression of a growth-promoting oncogene Ras or Myb in cells induces expression of the death suppressor Bcl-2 expression (Kinoshita et al., 1995; Grassilli et al., 1999). Recent work has also suggested involvement of apoptosis regulators in cell cycle progression.

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**ABBREVIATIONS:** PARP, poly(ADP-ribose) polymerase; VP-16, etoposide; SV40, simian virus 40; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; p21, the cyclin-dependent kinase inhibitor Cip1 or Waf-1.

For example, overexpression of the apoptosis inhibitor Bcl-2 delayed entry of activated T cells into S phase (O'Reilly et al., 1996) while overexpression of the apoptosis inducer Bax increased the G<sub>1</sub> to S transition (Brady et al., 1996). In addition, overexpression of Bcl-2 in other cell systems either inhibited the transition of G<sub>0</sub> to S or accelerated cycling cells exit into quiescent stage (Vairo et al., 1996). However, how endogenous Bcl-2 protein and mRNA are regulated during the cell cycle and how Bcl-2 protein regulates chemoresistance of tumor cells remain largely unknown.

In the current study, we measured levels of several Bcl-2 family proteins during the cell cycle progression using synchronized cells. We found that in all the tested five cell lines, expression of Bcl-2 protein peaked in the G<sub>1</sub> phase, whereas levels of Bax protein remained relatively unchanged in most of the cell lines. There were also little changes in levels of Bcl-X<sub>L</sub>, Bcl-X<sub>S</sub>, and Bak proteins during the cell cycle in Jurkat T cells. In addition, expression of Bcl-2 mRNA was also G<sub>1</sub> phase-specific, whereas the level of Bcl-2 degradation activity was constitutive. Furthermore, S phase cells expressing low levels of Bcl-2 protein were much more sensitive to apoptosis induction than G<sub>1</sub> cells expressing high levels of Bcl-2 protein, and Bcl-2 overexpression completely blocked the S phase-specific sensitivity to induced apoptotic cell death.

## Experimental Procedures

**Materials.** RPMI 1640, penicillin, and streptomycin were purchased from Life Technologies, Inc. (Rockville, MD). Fetal calf serum, aphidicolin, etoposide (VP-16), cisplatin, staurosporin, propidium iodide, RNase A, glucose, and salicylic acid were from Sigma Chemical Co. (St. Louis, MO). Monoclonal anti-human Bcl-2 antibody was obtained from Dako Co. (Glostrup, Denmark); polyclonal antibodies to human Bax and actin were from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal antibodies to human Bcl-X<sub>L</sub> and Bcl-X<sub>S</sub>, and monoclonal antibodies to human Bak and Caspase-3 were from Oncogene Research Products (Cambridge, MA); monoclonal antibodies to human p53 and p21<sup>Cip1</sup> were from Pharmingen (San Diego, CA); polyclonal antibody to human PARP was from Boehringer Mannheim (Indianapolis, IN). Anti-mouse IgG-horseradish peroxidase and anti-rabbit IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology. L-[<sup>35</sup>S]Methionine was from Amersham (Piscataway, NJ) and [<sup>32</sup>P]dCTP was from Boehringer Mannheim.

**Cell Culture, Synchronization, and Treatment.** Jurkat T cells transfected with bcl-2 cDNA (Bcl-2) or pcDNA3.0 alone (Neo) were gifts from Dr. Hong-gong Wang (Moffitt Cancer Center and Research Institute, Tampa, FL). MCF-7 cells, human Jurkat T cells, and Jurkat T cells overexpressing the human Bcl-2 oncoprotein or the vector alone were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Normal (WI-38) and simian virus 40 (SV40)-transformed (VA-13) human fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. All cells were maintained in a 5% CO<sub>2</sub> atmosphere at 37°C.

Cells were synchronized in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle by serum starvation. Briefly, exponentially grown cells were cultured in the serum-free medium for 72 (for MCF-7 cells) or 96 h (for WI-38 and VA-13 cells), followed by restimulating the cells to proliferate by addition of the serum. Cells were synchronized in the G<sub>1</sub>/S boundary by aphidicolin. Cells were incubated for 24 h with aphidicolin at a final concentration of 3 (for Jurkat T cells, Neo, and Bcl-2 cells) or 5 µg/ml (for MCF-7, WI-38, and VA-13 cells), followed by wash with

PBS and reculture in the growth medium. At each time point, one-third of the cells were harvested for Western blotting assay, one-third for cell cycle analysis, and one-third were treated for 3 or 24 h with either etoposide (50 µM/ml), cisplatin (20 µM/ml), or staurosporin (1 µM/ml). After each treatment, total cell populations (or a mixture of detached and attached cells) were collected, and used for assaying apoptotic cell death (see below).

**Cell Cycle Analysis.** Cell cycle analysis based on DNA content was performed as follows. Cells were harvested, counted, and washed twice with PBS. Cells ( $5 \times 10^6$ ) were then suspended in 0.5 ml of PBS, pipetted, and fixed in 5 ml of 70% ethanol for at least 2 h at -20°C. Cells were centrifuged, resuspended in 1 ml of propidium iodide staining solution (50 µg propidium iodide, 1 mg RNase A, and 1 mg of glucose per ml of PBS) and incubated at room temperature for 30 min. The cells were then analyzed with FACScan (Becton Dickinson Immunocytometry, Mountain View, CA) and ModFit LT cell cycle analysis software (Verity Software, Topsham, ME). The cell cycle distribution is shown as the percentage of cells containing G<sub>1</sub>, S, G<sub>2</sub>, and M DNA judged by propidium iodide staining. The apoptotic population (Ap) is the percentage of cells with <G<sub>1</sub> DNA content.

**Whole-Cell Extract and Western Blot Assay.** To prepare a whole cell extract, cells were lysed in a protein lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40) containing a freshly added cocktail of protease inhibitors (Erickson et al., 1998). The lysates were centrifuged at 20,000g for 30 min and the supernatant was collected. Equal amounts of protein (30–60 µg) were resolved by SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using a SemiDry Transfer System (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat dry milk in PBS-Tween (v/v, 0.2%) for 1 h at room temperature and then incubated overnight at 4°C with the specific antibody to Bcl-2 (1:500), Bax (1:300), Bcl-X<sub>L</sub> (1:100), Bcl-X<sub>S</sub> (1:100), Bak (1:100), Caspase-3 (1:500), actin (1:500), p53 (1:500), p21 (1:500), or PARP (1:3000). The membrane was washed, blotted with secondary antibody conjugated with horseradish peroxidase (1:2000) at room temperature for 1 h, and then washed again. The protein bands were visualized with the enhanced chemiluminescence system (Amersham) according to the manufacturer's instructions.

**Human bcl-2 cDNA Cloning and Bcl-2 Protein Degradation Assay.** Human, full-length, bcl-2α cDNA was amplified by reverse transcription-polymerase chain reaction using total RNA derived from Jurkat T cells. The primer pairs used for reverse transcription-polymerase chain reaction are: 5'-TACTCGAGAAGGATGGCG-CACGCTGGGA-3' (forward) and 5'-GCAAGCTTCTTCACTTGTC-GCTCAGA-3' (reverse). The obtained bcl-2 cDNA was confirmed by sequencing and cloned into pcDNA3.1(-) (Invitrogen, Carlsbad, CA). <sup>35</sup>S-labeled Bcl-2 protein was prepared with pcDNA3.1(-)-bcl-2 as a template by coupled in vitro transcription/translation using TNT-coupled reticulocyte lysate systems (Promega, Madison, WI) according to the manufacturer's instructions. To prepare a protein extract for Bcl-2 degradation assay, cells were lysed by Dounce homogenization in a buffer containing 20 mM HEPES, pH 7.4, 1.5 mM MgCl<sub>2</sub>, 5 mM KCl, and 1 mM dithiothreitol. The lysates were centrifuged at 20,000g for 30 min and the supernatants were collected. For the Bcl-2 degradation assay, <sup>35</sup>S-labeled Bcl-2 (1 µl) was incubated with 70 µg of the above cell extracts in an assay buffer (10 mM HEPES, pH 7.4, 5 mM MgCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>, 1 mM dithiothreitol, 0.1 mg/ml creatine kinase, 100 mM creatine phosphate, and 5 mM ATP) for 4 h at 37°C. The reactions were stopped by the addition of the same volume of 2× SDS sample buffer. After electrophoresis, the gel was treated with 1 M sodium salicylate and dried, followed by autoradiography.

**Northern Blot Assay.** Total RNA was extracted from  $5 \times 10^6$  synchronized cells using TRIzol (Life Technologies) according to the manufacturer's instructions. RNA was electrophoresed (30 µg/lane) in a 1.2% agarose gel, blotted onto a Zeta-Probe nylon membrane (Bio-Rad), and hybridized to <sup>32</sup>P-labeled bcl-2 cDNA according to

standard procedures (Hu et al., 1996). Bcl-2 mRNA was detected by autoradiography. After stripping residual radioactivity, membranes were hybridized with a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe. G3PDH mRNA was detected by autoradiography and used for normalization of RNA loading and hybridization efficiency. The bcl-2 and G3PDH cDNA probes were radiolabeled with [<sup>32</sup>P]dCTP using random primer DNA labeling kit (Boehringer Mannheim).

**DNA Fragmentation Assay.** After drug treatment, cells were harvested, washed twice with ice-cold PBS, resuspended in a DNA lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA, 1% SDS, and 0.5 mg proteinase K), and incubated for 24 h at 37°C. RNA was digested by adding 0.2 mg/ml of DNase-free RNase and incubating at 37°C for 1 h. DNA was precipitated by isopropanol, washed once with 75% ethanol, and dissolved in Tris-EDTA buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). Fifteen micrograms of DNA from each sample was subjected to electrophoresis on 1.2% agarose containing 0.5 µg/ml of ethidium bromide and visualized under UV light.

## Results

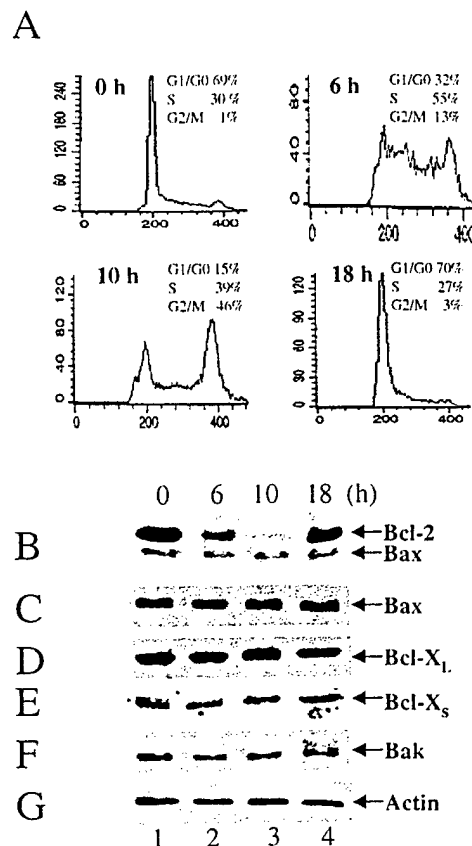
**G<sub>1</sub> Phase-Dependent Expression of Bcl-2 Protein in Human Tumor, Transformed, and Normal Cell Lines.** We investigated levels of Bcl-2 family proteins during the cell cycle progression. Human Jurkat T cells were synchronized at the G<sub>1</sub>/S boundary by using aphidicolin, an inhibitor of DNA polymerase α (Huberman, 1981), followed by removal of the drug and further incubation of the cells in fresh growth medium. At each time point, cells were harvested and used for measurement of the cell cycle distribution (by flow cytometry) and Bcl-2-like protein expression (by Western blot assay) (Fig. 1).

Treatment with aphidicolin for 24 h accumulated 69% of the Jurkat cells in G<sub>1</sub> and 30% in early S phase (Fig. 1A, 0 h). Immunoblot with a combination of Bcl-2 and Bax antibodies revealed a high level of Bcl-2 and a low level of Bax protein in these cells (Fig. 1B, lane 1). At 6 h after release from the aphidicolin block, the G<sub>1</sub> cell population was decreased by 37%, associated with 25% increase in S population and 12% increase in G<sub>2</sub>/M population (Fig. 1A). At this time, the Bcl-2 protein level was significantly decreased but Bax level remained unchanged (Fig. 1, B and C, lane 2 versus lane 1), resulting in a decrease in the Bcl-2/Bax ratio. At 10 h after removal of aphidicolin, both G<sub>1</sub> and S populations were decreased, associated with a marked increase in G<sub>2</sub>/M cell population (Fig. 1A). This was accompanied by a further decrease in the level of Bcl-2 protein (Fig. 1B, lane 3), indicating that Bcl-2 expression is not G<sub>2</sub>/M phase-dependent. At this time, the level of Bcl-2 became even lower than that of Bax that remained unchanged (Fig. 1, B and C, lane 3 versus lane 2). At 18 h, 70% of these cells had reentered into G<sub>1</sub> and 27% even into early S phase (Fig. 1, A), accompanied by reappearance of highly expressed Bcl-2 protein and a slight decrease in Bax expression (Fig. 1, B and C, lane 4 versus lane 3).

In contrast to the dramatic changes in expression of Bcl-2 during the cell cycle progression, levels of the antiapoptotic Bcl-X<sub>L</sub>, the proapoptotic Bcl-X<sub>S</sub>, and the proapoptotic Bak proteins changed only slightly during this process (Fig. 1, D–F). Therefore, Bcl-2 is the only member of the family we examined that demonstrated a significant cell cycle-dependent protein expression in Jurkat T cells. When Jurkat cells transfected with an empty vector (for the following Bcl-2

study) were treated by aphidicolin, followed by release for up to 24 h, we again observed a G<sub>1</sub> phase-associated Bcl-2 expression and an unchanged Bax expression (Fig. 2, A, D, and E, lanes 1–6).

To further study the G<sub>1</sub> phase-dependent expression of Bcl-2 protein, we synchronized several other human cell lines by either aphidicolin treatment or serum starvation. When human normal fibroblasts (WI-38) were treated with aphidicolin for 24 h, about 80% of the cells were accumulated in G<sub>1</sub> phase, which contained a very high level of Bcl-2 protein (Fig. 3A, lane 1). After the aphidicolin-pretreated WI-38 cells were incubated in fresh growth medium for up to 14 h, the G<sub>1</sub> population was dramatically decreased (by 32–48%), associated with a decrease of Bcl-2 expression to an undetectable level (Fig. 3A, lanes 2–4). After released for 18 to 24 h, the G<sub>1</sub> population of the cells was increased by 20 to 25%, accompanied by a significant increase in the levels of Bcl-2 protein (Fig. 3A, lanes 5 and 6). In contrast, very little change was observed in Bax protein levels in the treated WI-38 cells (Fig. 3A). When SV40-transformed WI-38 (VA-13; Fig. 3B) or breast carcinoma MCF-7 cells (Fig. 3C) were synchronized with aphidicolin and then released, the G<sub>1</sub> phase-dependent Bcl-2 protein expression was again observed.



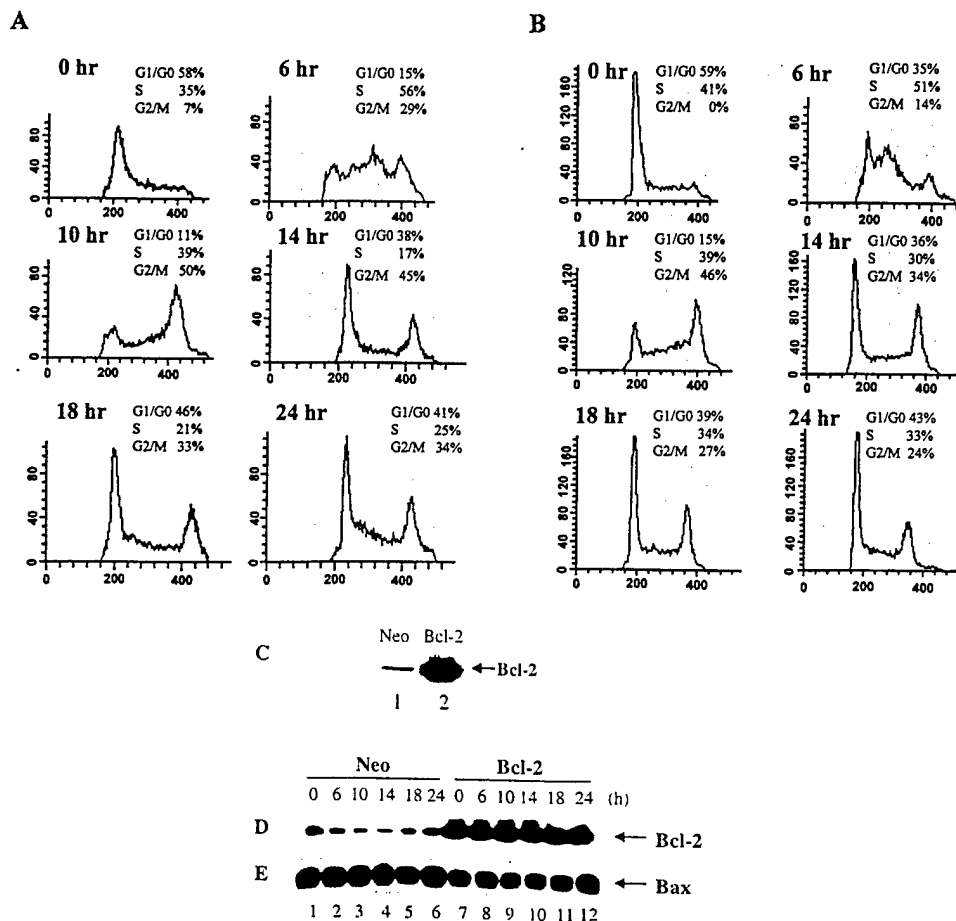
**Fig. 1.** Expression of Bcl-2 family members in human Jurkat T cells during the cell cycle progression. Human Jurkat T cells were treated with aphidicolin at 3 µg/ml for 24 h (0 h), followed by release for up to 18 h (see *Experimental Procedures*). At each time point, one-third of the cells were harvested for flow cytometry analysis of the cell cycle (A), one-third were for whole cell extraction and Western blot assay (B–G), and one-third were incubated with VP-16, followed by measurement of apoptosis (see Fig. 7). B, the filter was first incubated with a specific antibody to Bcl-2, rinsed, and then incubated with a Bax antibody. C, anti-Bax antibody alone. D to G, results using specific antibodies to Bcl-X<sub>L</sub>, Bcl-X<sub>S</sub>, Bak, and Actin, respectively.

To demonstrate that the observed  $G_1$  phase-associated Bcl-2 protein expression (Figs. 1–3) is not an artifact from the aphidicolin treatment, human breast cancer MCF-7 cells were synchronized in  $G_0/G_1$  phase by serum starvation and then restimulated to proliferate by the addition of fresh serum. About 95% of MCF-7 cells were synchronized in  $G_0/G_1$  phase by serum starvation for 72 h, which expressed high levels of Bcl-2 protein (Fig. 4A, lane 1). Addition of serum for 6 h resulted in ~10% of these cells across the  $G_1/S$  boundary, which was accompanied by a slight decrease in the level of Bcl-2 protein (Fig. 4A, lane 2 versus lane 1). After 16 to 20 h of stimulation, 50 to 60% of the originally synchronized  $G_1$  cells entered into S phase, and at these time points, the levels of Bcl-2 protein were significantly decreased (lanes 3 and 4). After 24 h, ~15% of cells entered into  $G_2/M$  phase, and the level of Bcl-2 protein still remained low (Fig. 4A, lane 5). When SV40-transformed WI-38 cells were serum-starved and then released, the  $G_1$  phase-dependent Bcl-2 expression was again detected (Fig. 4B). These data confirmed that expression of Bcl-2 protein is  $G_1$  phase-dependent.

We also measured levels of Bax in these experiments. Expression of Bax protein remained relatively constitutive during the cell cycle progression in aphidicolin-synchronized WI-38 (Fig. 3A) and VA-13 cells (data not shown from the experiment in Fig. 3B) as well as in serum starvation-synchronized VA-13 cells (Fig. 4B), which were similar to the results obtained from aphidicolin-synchronized Jurkat T (Fig. 1C) or Jurkat T cells transfected with a vector (Fig. 2E, lanes 1–6). In contrast to all of these four cell lines, expres-

sion of Bax in MCF-7 cells, synchronized by either aphidicolin treatment (Fig. 3C) or serum starvation (Fig. 4A), showed a cell cycle-dependent pattern. Low levels of Bax protein were detected in MCF-7 cells with high percentage of  $G_1$  population (Fig. 3C, lanes 1, 5, and 6, and Fig. 4A, lanes 1 and 2), whereas very high Bax expression was found in the cells containing high S,  $G_2$ , and M populations (Fig. 3C, lanes 2–4, and Fig. 4A, lanes 3–5).

MCF-7 cells express wild-type p53 gene (Runnebaum et al., 1991). It has been shown that p53 plays an essential role in down-regulation of Bcl-2 expression (Halder et al., 1994; Miyashita et al., 1994) and up-regulation of Bax transcription (Miyashita and Reed, 1995) in several cell systems. We then investigated whether the cell cycle-dependent expression of Bcl-2 and Bax correlated to any change in p53 levels in MCF-7 cells synchronized by either aphidicolin treatment (Fig. 3C) or serum starvation (Fig. 4A). Expression of p53 protein was low in  $G_1$  phase, but significantly increased when the cells progressed into S and  $G_2/M$  phases (Figs. 3C and 4A). Expression of the cyclin-dependent kinase inhibitor p21, a well-known downstream target of p53 (El-Deiry et al., 1993), was also cell cycle-dependent in these MCF-7 cells. More importantly, the periodic expression pattern of p21 was almost identical with that of p53 (Figs. 3C and 4A), suggesting that p53 is functional as a transcription factor under these conditions. Although the cell cycle-dependent pattern of Bax expression was similar to that of p53, the  $G_1$  phase-associated expression of Bcl-2 was inversely comparable with levels of p53 (Figs. 3C and 4A). These data are consistent



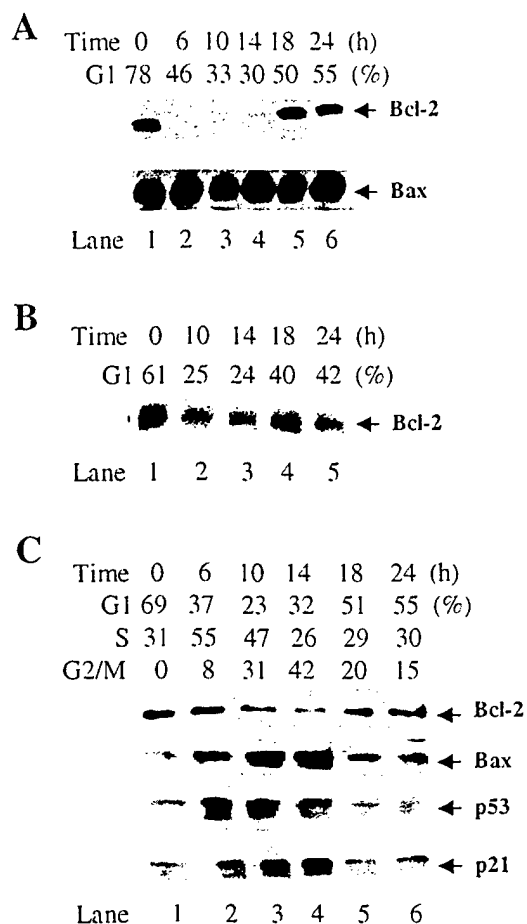
**Fig. 2.** Cell cycle-dependent expression of Bcl-2 and Bax protein in human Jurkat T cells transfected with an empty vector (Neo) or bcl-2 cDNA-containing vector (Bcl-2). Exponentially grown Neo- and Bcl-2-expressing Jurkat cells (C) were treated with aphidicolin at 3  $\mu$ g/ml for 24 h (0 h), followed by release for 6, 10, 14, 18, or 24 h, as indicated. At each time point, one-third of the cells were harvested for cell cycle analysis (A, B), one-third were for Western blot assay using specific antibodies to Bcl-2 (D) or Bax (E), and one-third were incubated with VP-16 or staurosporin, followed by measurement of apoptosis (see Fig. 8).

with the hypothesis that p53 up-regulates Bax and down-regulates Bcl-2 during the cell cycle progression of MCF-7 cells. Interestingly, although normal human WI-38 cells also contain wild-type p53 gene, these cells expressed unchanged levels of p53 during the cell cycle (data not shown from the experiment in Fig. 3A).

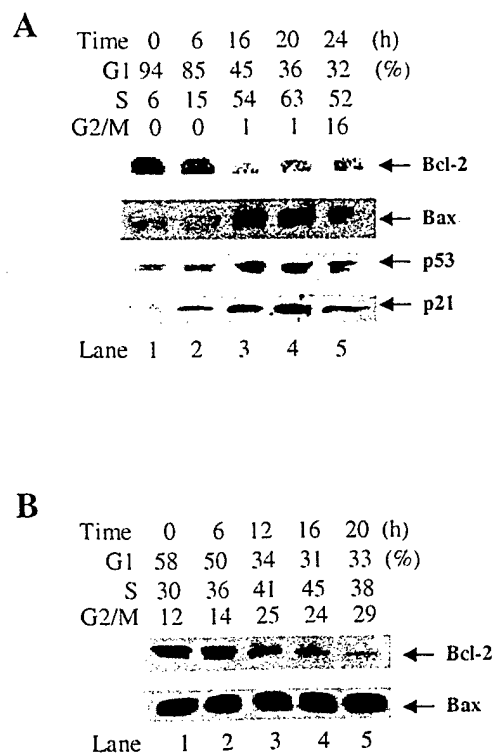
**G<sub>1</sub> Phase-Dependent Bcl-2 Protein Expression Is Regulated on the Level of Bcl-2 mRNA but Not Bcl-2 Proteolysis.** The high expression of Bcl-2 protein in G<sub>1</sub> phase could be caused by either a low level of a Bcl-2 proteolytic activity or a high level of Bcl-2 mRNA expression. To investigate these two possibilities, we measured levels of Bcl-2 degradation activity and Bcl-2 mRNA expression in the cell cycle. Aphidicolin-synchronized human Jurkat T (Fig. 5) and MCF-7 cells (Fig. 6) were used for extraction of proteins (for proteolysis assay) and mRNAs (for Northern blot assay). A [<sup>35</sup>S]methionine-labeled Bcl-2 protein, generated by *in vitro* transcription and translation of a full-length human bcl-2α cDNA, was used as a substrate in a cell-free Bcl-2 proteolysis assay (see Fig. 6A, lane 1). When incubated with a protein extract prepared from the aphidicolin-treated Jurkat cells, a portion of the labeled Bcl-2 was cleaved, as evidenced by appearance of a labeled band with a faster mobility

(indicated by an arrowhead; compare Fig. 5A, lane 1, with Fig. 6A, lane 1), suggesting the presence of a Bcl-2 proteolytic activity in the G<sub>1</sub>/S cells. The same Bcl-2 protein cleavage product was observed in a similar intensity when protein extracts prepared from other time points were used (Fig. 5A), which corresponded to different phases of the cell cycle (see Figs. 1A and 2A). This result indicates that the level of the Bcl-2 protein cleavage activity is constitutive during the cell cycle progression and, therefore, should not be responsible for the G<sub>1</sub> phase-dependent expression of Bcl-2 protein (Figs. 1–4).

When the RNA samples, prepared from the synchronized Jurkat T cells, were analyzed by Northern blot assay using a specific bcl-2 cDNA probe, a cell cycle-dependent pattern of Bcl-2 mRNA expression was observed (Fig. 5B). Jurkat cells, which were accumulated at G<sub>1</sub>/S by aphidicolin treatment (see 0 h in Figs. 1A and 2A), expressed a high level of Bcl-2 mRNA (Fig. 5B, lane 1). At 6 h after removing aphidicolin, ~40% of the original G<sub>1</sub> cells entered into S, G<sub>2</sub>, and M phase (Figs. 1A and 2A), associated with a decrease in the level of Bcl-2 mRNA (Fig. 5B, lanes 2 versus 1). At 10 and 14 h, ~50% of cells were accumulated in G<sub>2</sub>/M phase (Figs. 1A and 2A), where the lowest level of Bcl-2 mRNA was detected (Fig. 5B, lanes 3, 4). At 18 h and 24 h, the cells reentered into G<sub>1</sub> and later G<sub>1</sub>/S phase (Figs. 1A and 2A), where the levels of Bcl-2 mRNA were significantly increased (Fig. 5B, lanes 5, 6). By stripping and rehybridizing the filter with a human G3PDH probe, a constitutive expression of G3PDH mRNA was observed (Fig. 5B). Therefore, expression of endogenous Bcl-2



**Fig. 3.** G<sub>1</sub> phase-specific expression of Bcl-2 protein in aphidicolin-synchronized cells. Exponentially grown WI-38 (A), VA-13 (B), or MCF-7 cells (C) were treated with aphidicolin at 5 μg/ml for 24 h (lane 1, 0 h), followed by release for up to 24 h, as indicated. At each time point, cells were harvested for cell cycle analysis (indicated by percentage of cells in each phase of the cell cycle) and Western blotting using specific antibodies to Bcl-2, Bax, p53, or p21 protein.



**Fig. 4.** G<sub>1</sub> phase-specific expression of Bcl-2 protein in serum starvation-synchronized cells. Exponentially grown MCF-7 cells were cultured in the serum-free medium for 72 h (0 h in A) or exponentially grown VA-13 cells were cultured in the serum-free medium for 96 h (0 h in B), followed by addition of the serum for up to 24 h as indicated. At each time point, cells were harvested for cell cycle analysis (indicated by percentage of cells in each phase of the cell cycle) and Western blotting using specific antibodies to Bcl-2, Bax, p53, or p21 protein.

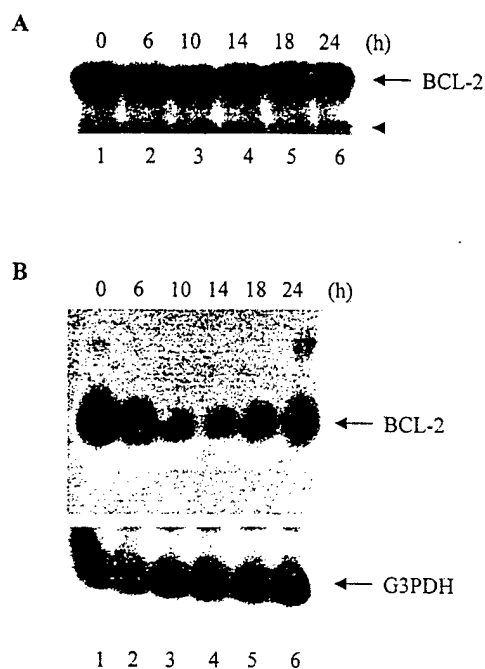
mRNA in Jurkat T cells is  $G_1$  phase-specific. The cell cycle-dependent pattern of Bcl-2 mRNA (Fig. 5B) was almost identical with that of Bcl-2 protein (Fig. 2D, lanes 1–6).

We also measured levels of Bcl-2 proteolytic enzyme activity and Bcl-2 mRNA in MCF-7 cells synchronized by aphidicolin (Fig. 6). Again, we observed constitutive levels of the Bcl-2 cleavage activity (Fig. 6A) and  $G_1$  phase-specific expression of Bcl-2 mRNA (Figs. 6B versus 3C). The cell cycle dependent pattern of Bcl-2 mRNA expression was again very similar to that of Bcl-2 protein under these conditions (compare Figs. 6B and 3C). From these data, we concluded that the  $G_1$  phase-specific expression of Bcl-2 mRNA is responsible for the  $G_1$  phase-dependent expression of Bcl-2 protein during the cell cycle.

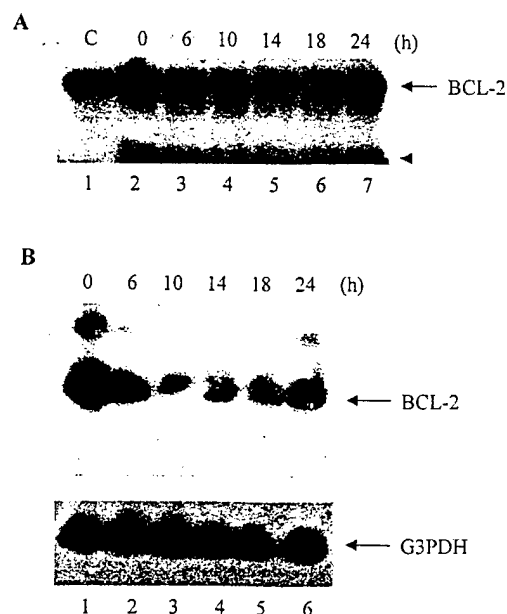
**S Phase Cells Containing Low Bcl-2 Levels Are Most Sensitive to Apoptosis Induction.** Many human cancers are resistant to apoptosis induced by chemotherapy, which is at least partially caused by overexpression of the Bcl-2 oncoprotein (Reed, 1995; Simonian et al., 1997). We hypothesized that the periodic expression of Bcl-2 protein should influence tumor cellular chemosensitivity in a cell cycle-dependent manner. To test this idea, human Jurkat, Neo, MCF-7, or VA-13 cells were synchronized by either exposure to aphidicolin, followed by release, or withdrawal of serum, followed by addition of fresh medium, as described in Figs. 1 to 4. At

each time point, an aliquot of the synchronized cells was incubated for additional 3 or 24 h with either a chemotherapeutic agent (VP-16 or cisplatin) or the kinase inhibitor staurosporin (Tamaoki, 1991). This was followed by collecting total cell population (for MCF-7 and VA-13 cells, the detached and the attached fractions were combined) and measuring cell death by apoptotic peak, PARP cleavage, Caspase-3 processing/activation, DNA fragmentation (from the 3-h treatment) and cell viability (from the 24 h treatment).

After the aphidicolin-treated Jurkat T cells (containing 69% of  $G_1$  population and a high level of Bcl-2 protein; Fig. 1, A and B) were exposed to VP-16 for 3 h, no apoptosis occurred, as demonstrated by lack of pre- $G_1$  population (Fig. 7A) and apoptosis-specific p85/PARP fragment, p17/Caspase-3 fragment (Fig. 7, B and C, lane 1) and DNA ladder (data not shown but see Fig. 8B). However, progression of these cells from  $G_1$  to S/ $G_2$ /M phases was apparent after the treatment (compare 0 h in Fig. 7A with 0 h in Fig. 1A), indicating that 3-h exposure to VP-16 had little or no effect on the cell cycle progression. When VP-16 was incubated for 3 h with the Jurkat cells, which were released from the aphidicolin block for 6 and 10 h and that contained decreased  $G_1$  population and Bcl-2 expression (Fig. 1, A and B), apoptosis occurred, as evidenced by 20 to 30% increase in the pre- $G_1$  apoptotic population (Fig. 7A) and a significant amount of the p85/PARP cleavage fragment, the active p17/Caspase-3 fragment (Fig. 7, B and C, lanes 2 and 3) and fragmented DNA (data not shown, but see Fig. 8B). Along with increased pre- $G_1$  population, the 3-h VP-16 treatment also decreased cell populations mainly from S/ $G_2$ /M phases of the cell cycle (compare 6 h and 10 h in Fig. 7A with 6 h and 10 h in Fig. 1A). No



**Fig. 5.** Levels of Bcl-2 proteolytic activity and mRNA during the cell cycle progression in human Jurkat T cells. Human Jurkat T cells were treated by aphidicolin for 24 h (0 h), followed by release for up to 24 h. At each time point, protein (A) and mRNA (B) samples were prepared. A, Bcl-2 proteolytic activity assay.  $^{35}$ S-labeled Bcl-2 protein (1  $\mu$ l; see lane 1 in Fig. 6A) was incubated at 37°C for 4 h with 70  $\mu$ g of a protein extract prepared from aphidicolin-synchronized cells, followed by SDS-polyacrylamide gel electrophoresis and autoradiography. An arrow and an arrowhead, respectively, indicate the uncleaved Bcl-2 protein and a cleavage fragment. B, Northern blot assay. The extracted total RNA samples were electrophoresed, blotted, and hybridized to a  $^{32}$ P-labeled bcl-2 cDNA. Levels of Bcl-2 mRNA were detected by autoradiography (upper). After stripping, the membrane was hybridized with a G3PDH cDNA probe. The G3PDH mRNA was measured for normalization of RNA loading (lower). The Bcl-2 and G3PDH mRNAs are approximately 6.5 and 1.4 kilobase pairs, respectively.



**Fig. 6.** Levels of Bcl-2 proteolytic activity and mRNA during the cell cycle progression in human breast carcinoma MCF-7 cells. MCF-7 cells were treated by aphidicolin for 24 h (0 h), followed by release for up to 24 h. At each time point, protein (A) and mRNA (B) samples were prepared. A, Bcl-2 proteolytic activity assay.  $^{35}$ S-labeled Bcl-2 protein (as a control, C, in lane 1) was incubated at 37°C for 4 h with 70  $\mu$ g of a protein extract prepared from aphidicolin-synchronized MCF-7 cells. An arrow and an arrowhead, respectively, indicate the uncleaved and cleaved Bcl-2. B, Northern blot assays. The mRNA levels of Bcl-2 (top) and G3PDH (bottom) are shown.



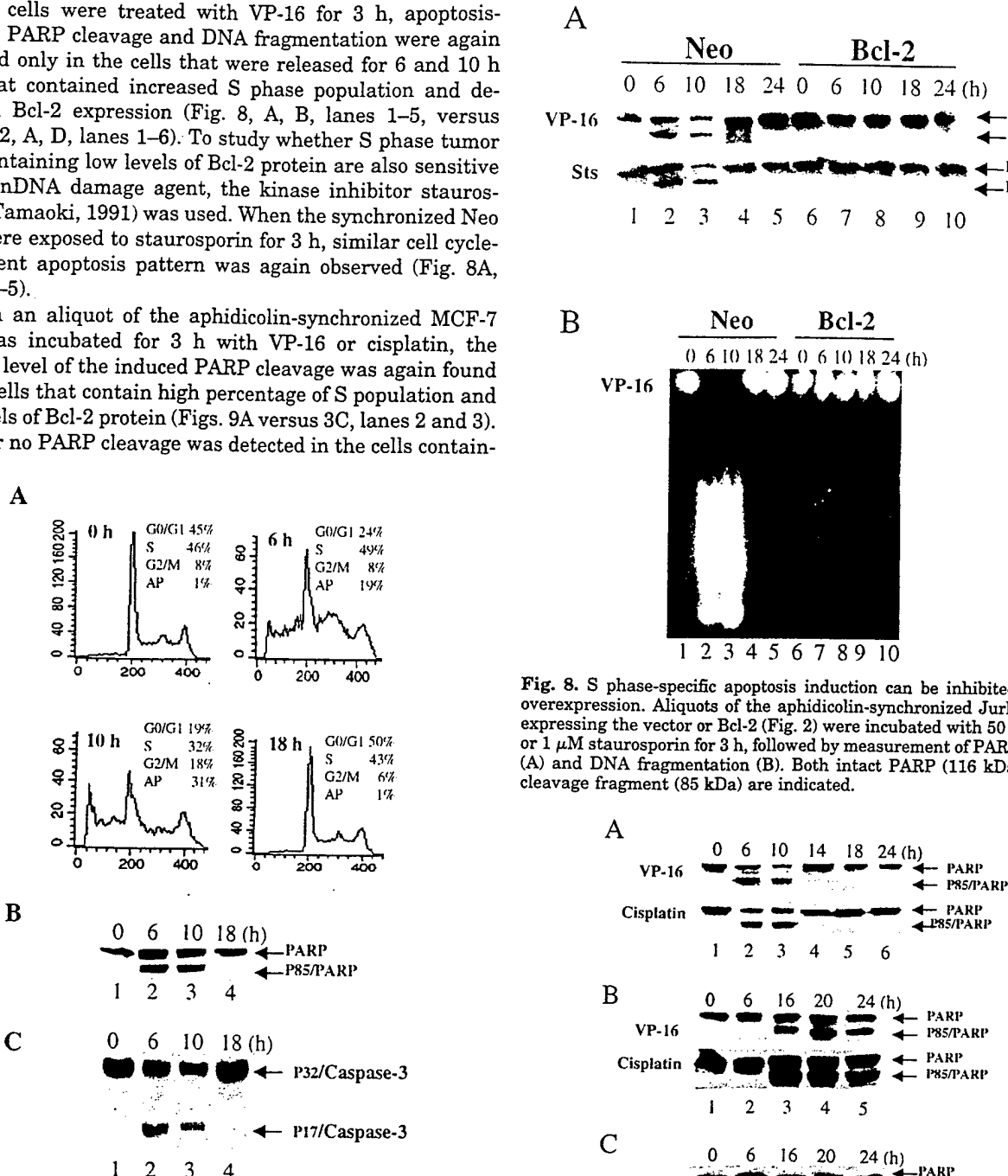
apoptotic cell death was observed when VP-16 was added into the aphidicolin-pretreated cells that had been released for 18 h (Fig. 7, A, B, and C, lane 4), which contained significantly increased G<sub>1</sub> population and Bcl-2 protein expression (Fig. 1, A and B). The 3-h VP-16 treatment did not block the cell cycle progression in these cells, either (compare 18 h in Figs. 7A and 1A).

When the aphidicolin-synchronized vector-transfected Jurkat T cells were treated with VP-16 for 3 h, apoptosis-specific PARP cleavage and DNA fragmentation were again detected only in the cells that were released for 6 and 10 h and that contained increased S phase population and decreased Bcl-2 expression (Fig. 8, A, B, lanes 1–5, versus Figure 2, A, D, lanes 1–6). To study whether S phase tumor cells containing low levels of Bcl-2 protein are also sensitive to a nonDNA damage agent, the kinase inhibitor staurosporin (Tamaoki, 1991) was used. When the synchronized Neo cells were exposed to staurosporin for 3 h, similar cell cycle-dependent apoptosis pattern was again observed (Fig. 8A, lanes 1–5).

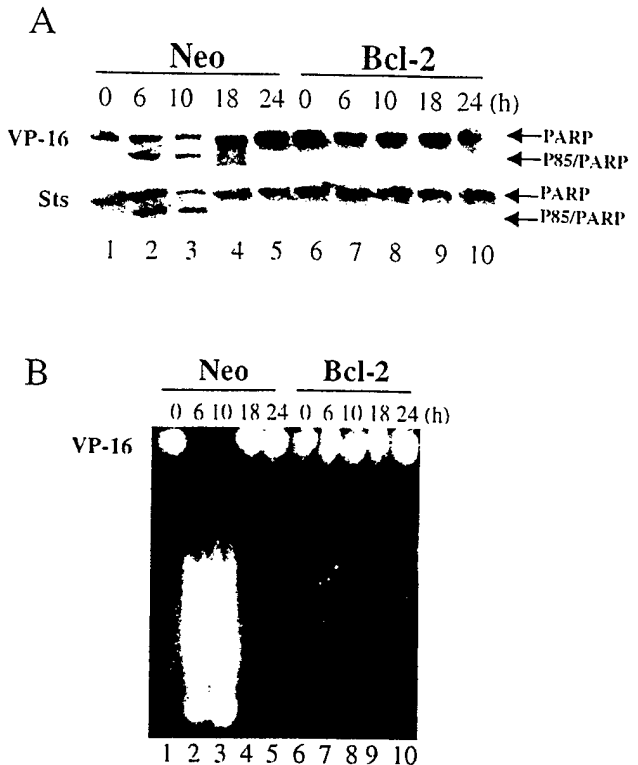
When an aliquot of the aphidicolin-synchronized MCF-7 cells was incubated for 3 h with VP-16 or cisplatin, the highest level of the induced PARP cleavage was again found in the cells that contain high percentage of S population and low levels of Bcl-2 protein (Figs. 9A versus 3C, lanes 2 and 3). Little or no PARP cleavage was detected in the cells contain-

ing high G<sub>1</sub> populations and high Bcl-2 levels (Figs. 9A versus 3C, lanes 1, 5, and 6). No PARP cleavage was found in the cells that contained a low percentage of S population and also a low level of Bcl-2 (Figs. 9A versus 3C, lane 4), suggesting that the decrease in Bcl-2 expression is not sufficient to sensitize tumor cells (see under *Discussion*).

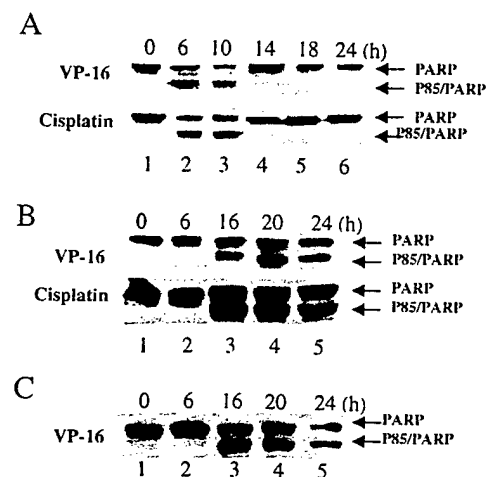
When aliquots of serum starvation-synchronized MCF-7



**Fig. 7.** Cell cycle-dependent apoptosis induction by VP-16 in human Jurkat T cells. Human Jurkat T cells were synchronized as described in Fig. 1. At each time point after the release from aphidicolin, a portion of cells was treated with 50  $\mu$ M VP-16 for additional 3 h and were then harvested for measurement of cell cycle distribution and sub-G<sub>1</sub> population by flow cytometry (A), PARP cleavage (B), and Caspase-3 processing/activation (C). The intact PARP (116 kDa), a PARP cleavage fragment (85 kDa), pro-Caspase-3 (32 kDa), and an active form of Caspase-3 (17 kDa) are indicated.



**Fig. 8.** S phase-specific apoptosis induction can be inhibited by Bcl-2 overexpression. Aliquots of the aphidicolin-synchronized Jurkat T cells expressing the vector or Bcl-2 (Fig. 2) were incubated with 50  $\mu$ M VP-16 or 1  $\mu$ M staurosporin for 3 h, followed by measurement of PARP cleavage (A) and DNA fragmentation (B). Both intact PARP (116 kDa) and the cleavage fragment (85 kDa) are indicated.



**Fig. 9.** S phase-specific, apoptosis-associated PARP cleavage in solid tumor cell lines. Aliquots of (A) the aphidicolin-synchronized MCF-7 cells (from Fig. 3C) (A), the serum starvation-synchronized MCF-7 cells (from Fig. 4A) (B), or the serum starvation-synchronized VA-13 cells (Fig. 4B) (C) were incubated with 50  $\mu$ M VP-16 or 20  $\mu$ M cisplatin for 3 h, followed by collecting both detached and attached cells for assaying PARP cleavage.

(Fig. 4A) or VA-13 cells (Fig. 4B) were incubated with VP-16 or cisplatin, a tight correlation between induced apoptosis, cell cycle distribution and endogenous Bcl-2 protein levels was again obtained. When cells containing high percentages of S population and low levels of Bcl-2 protein (Fig. 4, A and B, lanes 3–5) were used, a 3-h drug treatment induced apoptosis-specific PARP cleavage (Fig. 9, B and C, lanes 3–5) and 24-h drug treatment induced loss of cell viability (60–85%). In contrast, when cells containing high  $G_1$  populations and high Bcl-2 protein levels (Fig. 4, A and B, lanes 1, 2) were used, much less cell death was detected: a 3-h treatment of these  $G_1$  cells induced no PARP cleavage (Fig. 9, B and C, lanes 1, 2), and a 24-h treatment induced loss of membrane permeability in a much lower percentage of cells (10–20%). These data suggest that tumor cell sensitivity to apoptosis induction is associated with both high S phase population and low Bcl-2 expression.

**Overexpression of Bcl-2 Protein Partially Inhibits the  $G_1$  to S Transition and Completely Blocks the S Phase-Specific Chemosensitivity.** To study the functional significance of the cell cycle-dependent Bcl-2 protein expression, we used human Jurkat T cells that were transfected with the human bcl-2 gene. Overexpression of Bcl-2 protein in these cells was confirmed by Western blot analysis (Fig. 2C). Both Bcl-2- and vector-expressing cells were synchronized by aphidicolin, followed by release. At each time point, one-third of the cells were used for measurement of cell cycle distribution (Fig. 2, A and B), one-third for determination of Bcl-2 and Bax protein expression (Fig. 2, D and E), and one-third were incubated with VP-16 or staurosporin for an additional 3 h, followed by measurement of PARP cleavage and DNA fragmentation (Fig. 8).

As reported previously (Mazel et al., 1996; O'Reilly et al., 1996), overexpression of Bcl-2 protein slowed down the  $G_1$  to S transition: after 6-h release from the aphidicolin block, only 24% of  $G_1$  population entered into S/ $G_2$ /M phases in Bcl-2-expressing cells (Fig. 2B, 6 h versus 0 h), compared with 43% in the vector-expressing cells (Fig. 2A, 6 h versus 0 h). More importantly, at each time point, addition of VP-16 or staurosporin into Bcl-2 cells failed to induce PARP cleavage and DNA fragmentation (Fig. 8, A and B, lanes 6–10 versus 1–5). This was not caused by different cell cycle distribution of two cell lines, which is supported by the following comparison. At 10 h after release from the aphidicolin block, both Bcl-2- and vector-expressing cells contained similar percentages of  $G_1$ , S, and  $G_2$ /M populations (Fig. 2, B versus A), but different levels of Bcl-2 protein (Fig. 2D, lanes 9 versus 3). Addition of VP-16 or staurosporin at this time induced apoptosis only in the vector-, but not in Bcl-2-, expression cells (Fig. 8, A and B, lane 3 versus lane 8). Therefore, overexpression of Bcl-2 protein blocks the tumor cell S phase-associated sensitivity to apoptosis induction. Our data suggest that the cell cycle-dependent expression of endogenous Bcl-2 protein at least contributes to the cell cycle-dependent regulation of tumor cell chemoresistance.

## Discussion

Recently, it has been reported that some cell-cycle regulators, such as p53, RB, E2F, and Myc, play a role in the process of apoptosis (Lee et al., 1993; Kastan et al., 1995; Adams and Kaelin, 1996; Dou, 1997; Thompson, 1998). In

addition, it has also been reported that overexpression of cell death regulator Bcl-2 or Bax influences cell cycle progression (Brady et al., 1996; Mazel et al., 1996; O'Reilly et al., 1996; Gil-Gomez et al., 1998). However, how Bcl-2 and Bax are regulated under physiological conditions and how their regulation affects tumor cell chemosensitivity are unclear. In the current study, we have reported the following novel findings. First, expression of endogenous Bcl-2 mRNA and protein is  $G_1$  phase-dependent in the cell cycle. Second, high Bcl-2-containing  $G_1$  cell population is much more resistant to apoptosis induction by chemotherapeutic agents or staurosporin than low Bcl-2-containing S phase population. Finally, overexpression of Bcl-2 partially prevented the  $G_1$  to S transition and, more importantly, completely inhibits the S phase-associated tumor cell sensitivity to induction of apoptosis.

By using five different mammalian cell lines (Jurkat T, Jurkat T-expressing the vector, WI-38, VA-13, and MCF-7) that had been synchronized by aphidicolin treatment or serum starvation, we observed that expression of Bcl-2 protein is  $G_1$  phase-specific (Figs. 1–4). The maximal expression of Bcl-2 protein seems to occur in mid- to late  $G_1$  phase, supported by the following evidence. First, an immediate reentry into  $G_1$  phase of the next cycle from  $G_2$ /M phase is not associated with an immediate increase in the level of Bcl-2 protein (14 h versus 10 h in Fig. 2, A and D, and Fig. 3C). In addition, after cells just crossed the  $G_1$ /S boundary, the level of Bcl-2 protein was further increased (24 h versus 18 h in Fig. 2, A and D). Furthermore, when more  $G_1$  cells entered into S, the levels of Bcl-2 protein were significantly decreased (Figs. 1–4). Because neither aphidicolin treatment nor serum starvation could synchronize all the cells, our data did not rule out the possibility that Bcl-2 is also expressed in early S phase.

Our results are consistent with previous reports in which Bcl-2 overexpression either delayed entry into S phase (O'Reilly et al., 1996) or accelerated cycling cells exit into quiescent stage (Vairo et al., 1996). In addition, our investigation further expanded the recent findings that Bcl-2 expression was increased upon serum withdrawal from H9C2 cardiac muscle cells (Wang et al., 1998) and that endogenous Bcl-2 protein levels correlated with sensitivity of human T cells to dexamethasone-induced apoptosis (Montani et al., 1999). It should also be noted that the half-life of Bcl-2 mRNA in B cells was found to be ~2.5 h (Seto et al., 1988) whereas the half-life of Bcl-2 protein in HL-60 cells was ~20 h (Blagosklonny et al., 1996). It seems that half-lives of Bcl-2 mRNA and protein in a cell might differ, both of which may also vary in different cell systems.

We investigated the molecular mechanism responsible for the  $G_1$  phase-dependent expression of Bcl-2 protein. We found that expression of Bcl-2 mRNA peaked in mid to late  $G_1$  (Fig. 5B versus Fig. 2A, and Fig. 6B versus Fig. 3C), suggesting that the periodic change in Bcl-2 protein expression is caused by the cell cycle-dependent change in Bcl-2 mRNA levels. This argument is further supported by the observation that levels of Bcl-2 cleavage activity were constitutive during the cell cycle progression (Figs. 5A and 6A).

It has been reported that expression of Bcl-2 can be down-regulated by the tumor suppressor protein p53 through either a negative responsive element in the bcl-2 gene (Miyashita et al., 1994) or an alternative unidentified mecha-

nism (Haldar et al., 1994). In the current study, three cell lines, Jurkat (Iwamoto et al., 1996), Jurkat expressing the vector and VA-13 (Zhu et al., 1991), contain inactive p53 protein. Therefore, a p53-independent mechanism must regulate the G<sub>1</sub> phase-specific expression of Bcl-2 mRNA and protein in these cells (Figs. 1–5). The current studies also included normal WI-38 and breast cancer MCF-7 (Runnebaum et al., 1991) cell lines, both of which contain wild-type p53 gene. Although levels of p53 remained unchanged in WI-38 cells (data not shown in the experiment presented in Fig. 3A), levels of p53, as well as that of p21, changed in a cell cycle-dependent manner in MCF-7 cells (Figs. 3C and 4A). Furthermore, there was an inverse relationship between the levels of Bcl-2 and p53 (as well as p21) in these cells (Figs. 3C and 4A), suggesting that in MCF-7 cells, p53 plays a role in down-regulating Bcl-2 expression in a cell cycle-dependent fashion.

A previous study also reported that p53 directly activates transcription of Bax gene (Miyashita and Reed, 1995). In MCF-7 cells, Bax expression was low in G<sub>1</sub> phase, dramatically increased in S, G<sub>2</sub>, and M phases, and decreased again in G<sub>1</sub> of the next cell cycle (Figs. 3C and 4A). This cell cycle-dependent pattern matched very well to that of p53 expression (Figs. 3C and 4A), suggesting that p53 up-regulates Bax in MCF-7 cells. Interestingly, levels of p53 in normal WI-38 cells remained unchanged, associated with constitutive expression of Bax (Fig. 3A and data not shown).

It has also been reported that intracellular levels of Bcl-2 in MCF-7 cells can be up-regulated by estrogen (Teixeira et al., 1995; Huang et al., 1997b), which is present in the fetal bovine serum. We found high levels of Bcl-2 in the serum-starved MCF-7 cells; these levels were dramatically decreased after addition of the fresh serum (Fig. 4A). These data are inconsistent with the involvement of estrogen in up-regulation of Bcl-2. In addition, the periodic changes of Bcl-2 expression were also observed in aphidicolin-synchronized MCF-7 cells (Fig. 3C), in which serum was present all the time. Therefore, the periodic Bcl-2 expression in our MCF-7 system is cell cycle-dependent and is probably not regulated by estrogen.

Most recent experiments have suggested that the ratio of proapoptotic Bcl-2 family members (such as Bax) to antiapoptotic Bcl-2 members (such as Bcl-2) determines whether a cell is committed to apoptotic death or not (Green and Reed, 1998). We have found that levels of Bcl-2 peaked in G<sub>1</sub> phase in all the tested cell lines, whereas levels of Bax remained relatively unchanged in all the cell lines except MCF-7 and levels of Bcl-X<sub>L</sub>, Bcl-X<sub>S</sub>, and Bak showed little change in synchronized Jurkat cells (Figs. 1–4). Our data suggest that the ratio of Bax to Bcl-2 changes in a cell cycle-dependent manner: low in G<sub>1</sub> and high in S (super high in S phased MCF-7 cells because of an increase in Bax protein expression). Furthermore, we found that the cell cycle-dependent Bax/Bcl-2 ratio is associated with periodic changes in tumor cell sensitivity to apoptosis induction. The S phase tumor cells were much more sensitive to apoptosis induction by chemotherapeutic agents or staurosporin than the G<sub>1</sub> phase tumor cells (Figs. 7–9 versus 1–4). In addition, S phased Jurkat cells overexpressing Bcl-2 protein, which had a decreased ratio of Bax to Bcl-2, became resistant to apoptosis induced by either VP-16 or staurosporin (Fig. 8A). Our data argue that the apoptotic commitment is regulated by the

ratio of proapoptotic Bcl-2 family members (such as Bax) to antiapoptotic Bcl-2 members (such as Bcl-2) in a cell cycle-dependent manner.

We also observed that Bcl-2 overexpression delayed the G<sub>1</sub> to S transition (Fig. 2), consistent with the previous reports (O'Reilly et al., 1996; Mazel et al., 1996). However, it is unclear whether this cell cycle-inhibitory effect is related to the anti-apoptotic function of Bcl-2. It has been suggested that these two inhibitory effects of Bcl-2 can be genetically separated (Huang et al., 1997a).

Many chemotherapeutic drugs kill cancer cells via induction of apoptosis. Previous studies have demonstrated that under certain experimental conditions, the G<sub>1</sub> to S transition of the cell cycle is the most susceptible point for some cell systems to implement a death program (Meikrantz et al., 1994; Meikrantz and Schlegel, 1995; Dou, 1997), although the involved molecular mechanisms remain unknown. Our current studies have suggested that G<sub>1</sub> phase-dependent expression Bcl-2 mRNA and protein is a possible molecular mechanism that is involved in the cell cycle-associated tumor cell chemosensitivity. We also noticed that cells containing low percentages of S population and low levels of Bcl-2 protein were resistant to the drug treatment (Figs. 3C and 9A, lane 4), suggesting that tumor cell chemosensitivity is controlled not only by decreased Bcl-2 levels but also by some other S phase-specific factor(s). This S phase factor may not be the tumor suppressor p53, because most of the cell lines used in this study contain inactive p53 protein. Although the nature of this S phase factor is unclear, the presence of the candidate S phase-specific factor controlling tumor cell chemosensitivity further argues that cell cycle regulation influences apoptotic death decision-making. Our future studies will focus on molecular mechanisms responsible for G<sub>1</sub> phase-specific Bcl-2 mRNA expression and the function of Bcl-2 protein in the cell cycle-dependent tumor chemoresistance.

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## Ester Bond-containing Tea Polyphenols Potently Inhibit Proteasome Activity *in Vitro* and *in Vivo*\*

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It has been discovered that proteasome inhibitors are able to induce tumor growth arrest or cell death and that tea consumption is correlated with cancer prevention. Here, we show that ester bond-containing tea polyphenols, such as (–)-epigallocatechin-3-gallate (EGCG), potently and specifically inhibit the chymotrypsin-like activity of the proteasome *in vitro* ( $IC_{50}$  = 86–194 nM) and *in vivo* (1–10  $\mu$ M) at the concentrations found in the serum of green tea drinkers. Atomic orbital energy analyses and high performance liquid chromatography suggest that the carbon of the polyphenol ester bond is essential for targeting, thereby inhibiting the proteasome in cancer cells. This inhibition of the proteasome by EGCG in several tumor and transformed cell lines results in the accumulation of two natural proteasome substrates, p27<sup>Kip1</sup> and I $\kappa$ B- $\alpha$ , an inhibitor of transcription factor NF- $\kappa$ B, followed by growth arrest in the G<sub>1</sub> phase of the cell cycle. Furthermore, compared with their simian virus-transformed counterpart, the parental normal human fibroblasts were much more resistant to EGCG-induced p27<sup>Kip1</sup> protein accumulation and G<sub>1</sub> arrest. Our study suggests that the proteasome is a cancer-related molecular target of tea polyphenols and that inhibition of the proteasome activity by ester bond-containing polyphenols may contribute to the cancer-preventative effect of tea.

Previous epidemiological studies have suggested that tea consumption may have a protective effect against human cancer (1–4). Recent animal studies have also demonstrated that green tea polyphenols could suppress the formation and growth of human cancers, including skin (5, 6), lung (7), liver (8), esophagus (9), and stomach (10). The major components of green and black tea include epigallocatechin-3-gallate (EGCG)<sup>1</sup>, epigallocatechin (EGC), epicatechin-3-gallate (ECG), epicatechin

(EC), and their epimers (see Fig. 1A). EGCG among those polyphenols has been most extensively examined because of its relative abundance and strong cancer-preventive properties (1, 11). EGCG has been shown to inhibit several cancer-related proteins, including urokinase (12), nitric-oxide synthase (13), telomerase (14), and tumor necrosis factor- $\alpha$  (15). However, nonphysiological concentrations of EGCG (*i.e.*, concentrations higher than those found in human serum after tea consumption) were used in some earlier studies. Whether one or more of these proteins are the real molecular targets of EGCG and other tea polyphenols under physiological conditions needs further investigations.

The 20S proteasome, a multicatalytic complex (700 kDa), constitutes the catalytic key component of the ubiquitous proteolytic machinery 26S proteasome (16–20). There are three major proteasomal activities: chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide hydrolyzing (PGPH) activities (16, 21). The ubiquitin-proteasome system plays a critical role in the specific degradation of cellular proteins (22), and two of the proteasome functions are to allow tumor cell cycle progression and to protect tumor cells against apoptosis (23). The chymotrypsin-like but not trypsin-like activity of the proteasome is associated with tumor cell survival (24, 25). Many cell cycle and cell death regulators have been identified as targets of the ubiquitin-proteasome-mediated degradation pathway. These proteins include p53 (26), pRB (27), p21 (28), p27<sup>Kip1</sup> (29), I $\kappa$ B- $\alpha$  (30), and Bax (31).

Here, we report for the first time that ester bond-containing tea polyphenols potently and selectively inhibit the proteasomal chymotrypsin-like but not trypsin-like activity *in vitro* and *in vivo*. Among the tea polyphenols examined, EGCG showed the strongest inhibitory activity against purified 20S proteasome, 26S proteasome of tumor cell extracts, and 26S proteasome in intact tumor cells. Furthermore, the inhibition of the proteasome *in vivo* was able to accumulate the natural proteasome substrates p27<sup>Kip1</sup> and I $\kappa$ B- $\alpha$  as well as induce the arrest of tumor cells in the G<sub>1</sub> phase. Finally, normal human WI-38 fibroblasts were more resistant to the EGCG treatment than their SV40-transformed counterpart.

### EXPERIMENTAL PROCEDURES

**Materials**—Highly purified tea polyphenols EGCG (>95%), ECG (>98%), EGC (>98%), EC (>98%), GCG (>98%), GC (>97%), CG (>98%), and C (>98%) were purchased from Sigma and used directly without further purification. A green tea extract was a gift from the Lipton Company (Englewood Cliffs, NJ) that contained 51.5% EGCG, 14.7% ECG, 8.3% EGC, 8.5% EC, 4.4% GCG, 2.4% GC, 1.6% C, and 1.6% caffeine. A black tea extract was also a gift from Lipton that contained 19.7% EGCG, 14.9% ECG, 0.9% EGC, 4.8% EC, 0.0% GCG, 0.5% GC, 2.0% C, and 1.2% caffeine. Purified 20S proteasome (*Methanosarcina thermophila*, Recombinant, *Escherichia coli*) and purified calpain I (human erythrocytes) were purchased from Calbiochem. Fluorogenic peptide substrates Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), benzoyloxycarbonyl (Z)-Leu-Leu-Glu-AMC (for the proteasomal PGPH activity), Suc-Leu-Tyr-AMC (for the calpain I

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<sup>1</sup> The abbreviations used are: EGCG, (–)-epigallocatechin-3-gallate; EGC, (–)-epigallocatechin; ECG, (–)-epicatechin-3-gallate; EC, (–)-epicatechin; GCG, (–)-gallocatechin-3-gallate; GC, (–)-gallocatechin; CG, (–)-catechin-3-gallate; C, (–)-catechin; AMC, 7-amido-4-methyl-coumarin; PGPH, peptidyl-glutamyl peptide-hydrolyzing; HPLC, high performance liquid chromatography; I $\kappa$ B- $\alpha$ , inhibitor of transcription factor NF- $\kappa$ B; p70, 70 kDa; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Z, benzoyloxycarbonyl.

activity), and Ac-Asp-Glu-Val-Asp-AMC (for the caspase-3 activity) were also obtained from Calbiochem, and Z-Gly-Arg-AMC (for the proteasomal trypsin-like activity) was from Bachem (King of Prussia, PA). The specific calpain inhibitor calpeptin and the specific caspase-3 inhibitor Ac-DEVD-CHO were obtained from Calbiochem. Monoclonal antibody to p27<sup>Kip</sup> was purchased from PharMingen (San Diego, CA), polyclonal antibodies to I $\kappa$ B- $\alpha$  and actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and polyclonal antibodies to ubiquitin were from Sigma.

**Cell Culture and Cell Extract Preparation**—Human Jurkat T and prostate cancer (LNCaP, PC-3) cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100  $\mu$ g/ml streptomycin. Human breast cancer MCF7 cells and normal (WI-38) and SV40-transformed (VA-13) human fibroblasts were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin, and streptomycin. All cells were maintained in a 5% CO<sub>2</sub> atmosphere at 37 °C. A whole cell extract was prepared as described previously (24). Cells were harvested, washed with phosphate-buffered saline twice, and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4 °C. Afterward, the lysates were centrifuged at 14,000  $\times$  g for 30 min, and the supernatants were collected as whole cell extracts.

**Inhibition of Purified 20S Proteasome Activity by Tea Polyphenols**—The chymotrypsin-like activity of purified 20S proteasome was measured as follows. 0.5  $\mu$ g of purified 20S proteasome was incubated with 20  $\mu$ M fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), for 30 min at 37 °C in 100  $\mu$ l of assay buffer (20 mM Tris-HCl, pH 8.0) with or without a tea polyphenol or tea extract. After incubation, the reaction mixture was diluted to 200  $\mu$ l with the assay buffer followed by a measurement of the hydrolyzed 7-amido-4-methyl-coumarin (AMC) groups using a VersaFluor<sup>TM</sup> Fluorometer with an excitation filter of 380 nm and an emission filter of 460 nm (Bio-Rad).

**Inhibition of the Proteasome Activity in Whole Cell Extracts by Tea Polyphenols**—A whole cell extract (3.5  $\mu$ g) of Jurkat T cells was incubated for 90 min at 37 °C with 20  $\mu$ M fluorogenic peptide substrates for various activities of the proteasomes, Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Leu-Glu-AMC, and Z-Gly-Gly-Arg-AMC, in 100  $\mu$ l of the assay buffer with or without EGCG or EGC. The hydrolyzed AMCs were quantified as described earlier.

**Inhibition of the Proteasome Activity in Intact Tumor Cells by Tea Polyphenols**—To measure the inhibition of proteasome activity in living tumor cells, Jurkat T ( $1 \times 10^5$  cells/ml/well), MCF-7, or PC-3 cells ( $1 \times 10^4$  cells/ml/well) were cultured in 24-well plates. These cells were first incubated for 12 h with various concentrations of EGCG, EGC,  $\beta$ -lactone, or LLnL followed by an additional 2-h incubation with the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC or Z-Gly-Gly-Arg-AMC. Afterward, the cell medium (200  $\mu$ l/sample) was collected and used for measurement of free AMCs.

**Assays for Calpain I and Caspase-3 Activities**—To measure the activity of calpain I, 3  $\mu$ g of purified calpain I was incubated with 40  $\mu$ M fluorogenic peptide calpain substrate, Suc-Leu-Tyr-AMC, for 30 min at 37 °C in 100  $\mu$ l of assay buffer (50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM  $\beta$ -mercaptoethanol, 5 mM CaCl<sub>2</sub>, and 0.1% CHAPS) with or without EGCG or the specific calpain inhibitor calpeptin. For caspase-3 activity assay, a Jurkat T cell extract (3.5  $\mu$ g) was incubated for 90 min at 37 °C with 20  $\mu$ M fluorogenic peptide substrate, Ac-Asp-Glu-Val-Asp-AMC, with or without EGCG or the specific caspase-3 inhibitor Ac-DEVD-CHO. After incubation, the reaction mixture was diluted to 200  $\mu$ l with the assay buffer, and the hydrolyzed AMCs were quantified as described above.

**Atomic Orbital Energy Analysis**—The electron density surface colored by nucleophilic susceptibility was created using the Cache Work-system version 3.2 (Oxford Molecular Ltd.). After geometrical optimization (using augmented MM3), the electron distribution between the highest occupied molecular orbital and the lowest unoccupied molecular orbital was evaluated, and a three-dimensional isosurface of susceptibility to nucleophilic attack (generated by an extended Huckel wave function) was calculated and superimposed over the molecule. A colored "bullseye" with a white center is characteristic of atoms that are highly susceptible to nucleophilic attack.

**Western Blot Analysis**—Jurkat T, LNCaP, WI-38, or VA-13 cells were treated with various concentrations of EGCG or EGC for indicated hours (see figure legends). To measure the changes in protein stability, Jurkat T cells were pretreated with 10  $\mu$ g/ml cycloheximide for 2 h (to inhibit translation) followed by incubation with EGCG or the proteasome inhibitor LLnV (for a positive control). This was followed by the

preparation of whole cell extracts. The enhanced chemiluminescence Western blot analysis was then performed using specific antibodies to p27<sup>Kip</sup>, I $\kappa$ B- $\alpha$ , ubiquitin, or actin as described previously (24).

**Flow Cytometry**—Cell cycle analysis based on DNA content was performed as follows. At each time point, cells were harvested, counted, and washed twice with phosphate-buffered saline. Cells ( $5 \times 10^6$ ) were suspended in 0.5 ml of phosphate-buffered saline, fixed in 5 ml of 70% ethanol for at least 2 h at -20 °C, centrifuged, resuspended again in 1 ml of propidium iodide staining solution (50  $\mu$ g of propidium iodide, 100 units of RNase A, and 1 mg of glucose/ml of phosphate-buffered saline), and incubated at room temperature for 30 min. The cells were then analyzed with FACScan (Becton Dickinson Immunocytometry, San Jose, CA) and ModFit LT cell cycle analysis software (Verity Software, Topsham, ME). The cell cycle distribution is presented as the percentage of cells containing G<sub>1</sub>, S, G<sub>2</sub>, and G<sub>2</sub>/M DNA content as judged by propidium iodide staining.

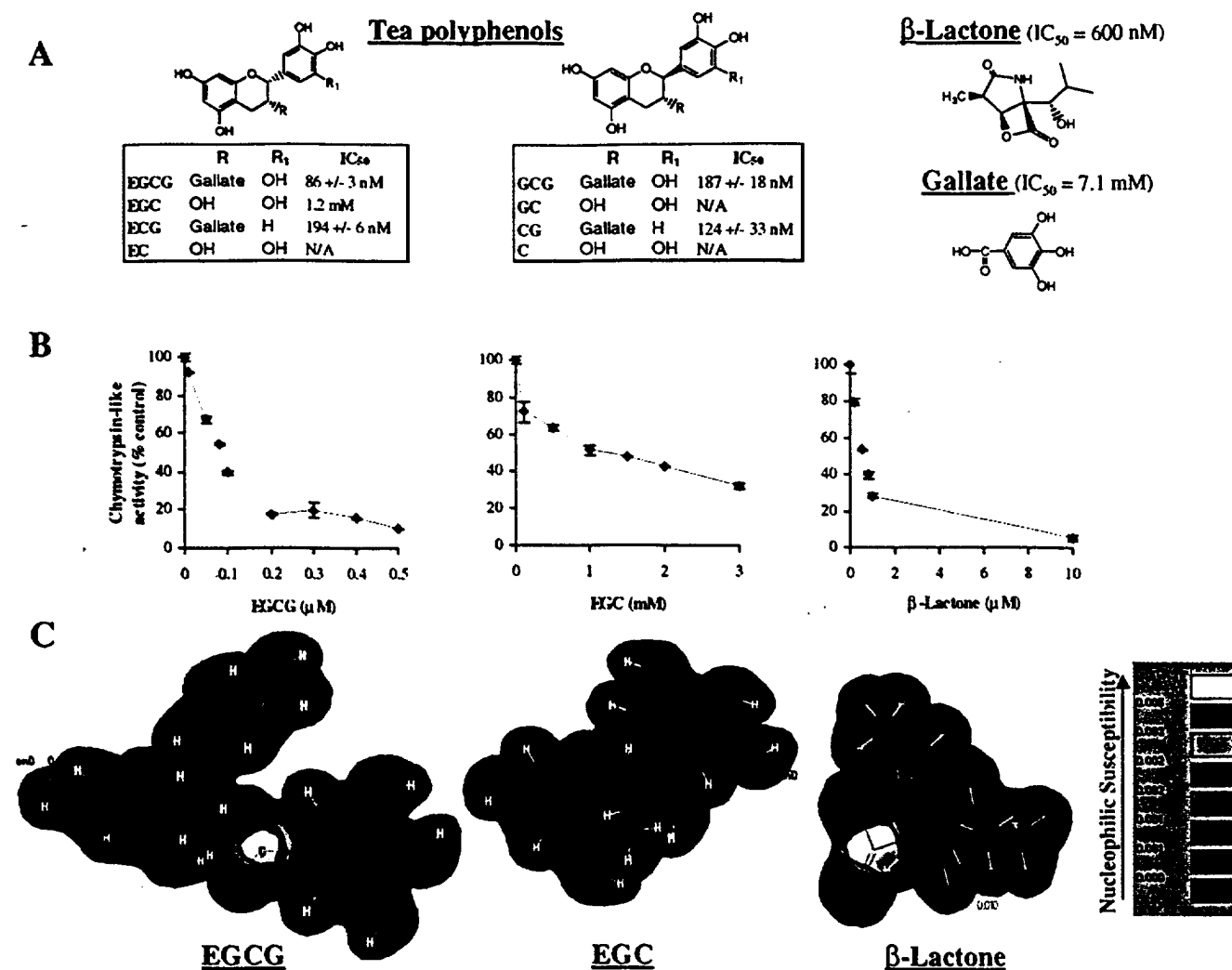
**High Performance Liquid Chromatography (HPLC) Analysis**—EGCG or EGC (1 mM) was incubated with either the purified 20S proteasome (45  $\mu$ g) or its buffer for the indicated hours at 37 °C in 100  $\mu$ l of a reaction buffer (20 mM Tris-HCl, 1 mM dithiothreitol, pH 7.2). After each reaction, the sample was filtered with a 0.45  $\mu$ m of nylon syringe filter (Nalge Co., Rochester, NY), and 20  $\mu$ l of filtered sample was injected to HPLC equipped with a C-18 reverse phase column (0.46  $\times$  25 cm, Separation Group, Hesperia, CA). The solvent system was 12% acetonitrile, 2% ethyl acetate, and 0.05% phosphoric acid, the flow rate was 1 ml/min, and the proteasome cleavage products were monitored at 280 nm. The standard controls also included gallic acid without incubation and the purified proteasome alone.

## RESULTS AND DISCUSSION

**Inhibition of Chymotrypsin-like Activity of Purified 20S Proteasome by Ester Bond-containing Tea Polyphenols**—It has been reported that lactacystin, when converted to its active form clasto-lactacystin  $\beta$ -lactone ( $\beta$ -lactone), is a highly specific and irreversible inhibitor of the proteasome (32–34). This  $\beta$ -lactone contains an ester bond (Fig. 1A) that is responsible for interacting with and inhibiting the proteasome (32–34). We noticed a similar ester bond present in several tea polyphenols including EGCG, EGC, GCG, and CG (Fig. 1A). We hypothesized that tea polyphenols containing ester bonds would inhibit the proteasome activity, whereas tea polyphenols without ester bonds would not. We tested this hypothesis by performing a cell-free proteasome activity assay in the presence of tea polyphenols. The chymotrypsin-like activity of purified 20S proteasome (the catalytic core of 26S proteasome) (22) was significantly inhibited by EGCG (Fig. 1B) whose IC<sub>50</sub> value was calculated to be 86 nM (Fig. 1A). In contrast, EGC (IC<sub>50</sub> = 1.2 mM) and gallic acid (IC<sub>50</sub> = 7.1 mM), the two moieties of EGCG linked by an ester bond, were 14,000- and 83,000-fold less potent than EGCG, respectively (Fig. 1, A and B). As a positive control,  $\beta$ -lactone also potently inhibited the proteasomal chymotrypsin activity (IC<sub>50</sub> = 600 nM in Fig. 1, A and B) (34). The shape of the inhibition curve of EGCG was similar to that of  $\beta$ -lactone (Fig. 1B).

Three other ester bond-containing tea polyphenols, EGC, GCG, and CG (Fig. 1A), were also found to be strong inhibitors of the chymotrypsin-like activity of the purified 20S proteasome (IC<sub>50</sub> values were 194, 187, and 124 nM, respectively). In contrast, all the corresponding polyphenols that do not contain ester bonds, EC, GC, and C (Fig. 1A), could not inhibit the proteasomal chymotrypsin-like activity. These results indicate that the ester bonds contained in tea polyphenols are essential for potent inhibition of the proteasomal chymotrypsin-like activity. Furthermore, a green or black tea extract, which contains significant portions of EGCG (51.5 and 19.7%, respectively) and EGC (14.7 and 14.9%, respectively, see under "Experimental Procedures"), also strongly inhibited the chymotrypsin-like activity of the 20S proteasome (IC<sub>50</sub> values were 0.1 and 0.3  $\mu$ g/ml, respectively).

The electrophilic ester bond carbon of  $\beta$ -lactone is responsible for its biological inhibition of the proteasome (32–34), sup-



**FIG. 1. Structure-activity relationships of tea polyphenols.** A, the structure and potency of polyphenols. IC<sub>50</sub> of each tea polyphenol toward the chymotrypsin-like activity of the purified 20S proteasome was measured as described under "Experimental Procedures." N/A indicates that the inhibitory activity of the corresponding polyphenol at 50 μM was <10%. B, concentration-dependent inhibition of the chymotrypsin-like activity of the purified 20S proteasome by EGCG, EGC, and β-lactone. C, the susceptibility of EGCG, EGC, and β-lactone to a nucleophilic attack was calculated as described under "Experimental Procedures."

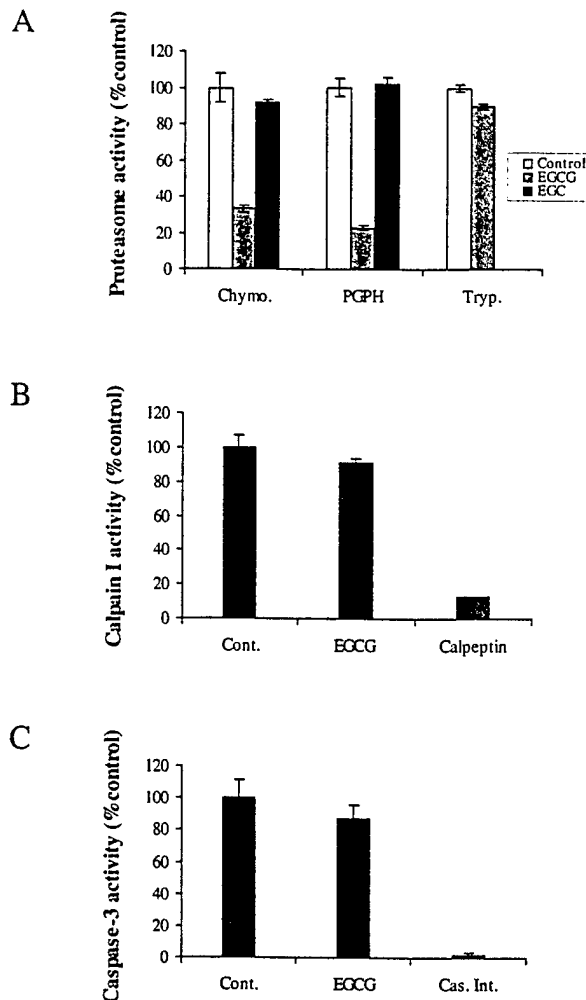
ported by previous studies using x-ray crystallography (16). When the atomic orbital energy was analyzed, the ester bond carbon of β-lactone showed a high susceptibility toward a nucleophilic attack with an arbitrary value of 1.1 (Fig. 1C). We then determined if the levels of nucleophilic susceptibility found in tea polyphenols correlate with their proteasome inhibitory activities. The ester bond carbon of EGCG was found to have the highest susceptibility toward a nucleophilic attack among all the other atoms with a value of 0.7, whereas the carbon with the highest nucleophilic susceptibility on EGC was found to have a low value of 0.2 (Fig. 1C). Similarly, a high nucleophilic susceptibility was found in other ester bond-containing polyphenols, ECG, GCG, and CG (all with values of 0.7), whereas low nucleophilic susceptibility was found in non-ester bond-containing polyphenols, EC, GC, and C (with values of 0.3, 0.2, and 0.3, respectively). Thus, the nucleophilic susceptibility of tea polyphenols correlated with their ability to inhibit the proteasome chymotrypsin-like activity. These data support the essential role of polyphenol ester bonds in the inhibition of the proteasome activity.

**Inhibition of the Proteasomal Chymotrypsin-like Activity in Tumor Cell Extracts and Intact Tumor Cells by EGCG**—We then tested if EGCG or EGC could inhibit the 26S proteasome activity in a tumor cell extract. We found that 10 μM EGCG

inhibited ~70% of the proteasomal chymotrypsin-like activity in a Jurkat T cell extract, whereas EGC at the same concentration had little effect (Fig. 2A). The addition of EGCG to the Jurkat cell extract also potentially inhibited another proteasomal activity, the PGPH activity, but did not affect the proteasomal trypsin-like activity (Fig. 2A). To investigate whether EGCG specifically inhibits the proteasome activity, its effects on other protease activities were examined. The activity of purified calpain I enzyme was inhibited by the specific calpain inhibitor calpeptin (35) but not EGCG (Fig. 2B). Similarly, a caspase-3-like activity in Jurkat T cell extract was blocked by the specific caspase-3 inhibitor Ac-DEVD-CMK (36) but not EGCG (Fig. 2C). It appears that EGCG selectively inhibits the proteasomal chymotrypsin (and PGPH) activity over other protease activities.

To determine whether EGCG could also inhibit the living cell proteasomal activity, Jurkat T cells were first incubated with various concentrations of EGCG or EGC followed by an additional incubation with a fluorogenic proteasome peptide substrate. Afterward, the cell medium was collected for the measurement of hydrolyzed products (free AMC). By performing this assay, we found that EGCG significantly inhibited the proteasomal chymotrypsin-like activity in intact Jurkat cells in a concentration-dependent manner (IC<sub>50</sub> = 18 μM), whereas

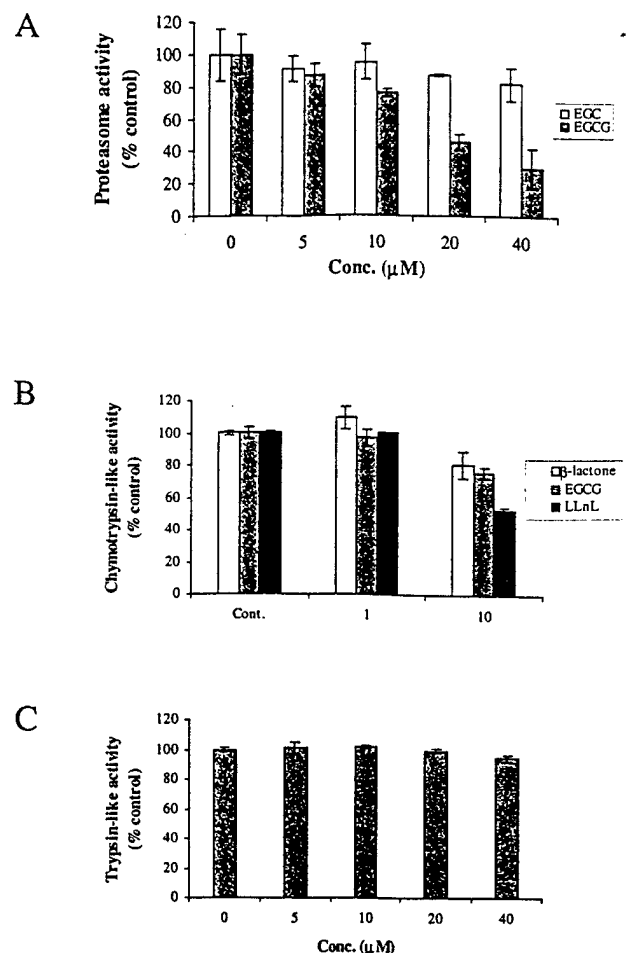




**FIG. 2. Specific inhibition of the proteasome activity by EGCG in Jurkat cell extracts.** A Jurkat cell extract (3.5  $\mu$ g/reaction) was incubated for 90 min with various fluorogenic peptide substrates for the proteasomal chymotrypsin-like, PGPH, trypsin-like activity (A), or caspase-3 activity (C), or a purified calpain I (3  $\mu$ g) was incubated for 90 min with a fluorogenic calpain substrate (B) in the absence or presence of EGCG (10  $\mu$ M), EGC (10  $\mu$ M), calpeptin (1  $\mu$ M), or Ac-DEVD-CHO (10  $\mu$ M) as indicated followed by the measurement of free AMC groups as described under "Experimental Procedures" ( $p < 0.05$ ).

EGC had a much less effect (Fig. 3A).

We noticed that the concentrations of EGCG needed to inhibit the proteasome activity in Jurkat cell extracts (Fig. 2A), and intact Jurkat cells (Fig. 3A) were much higher than were needed for the inhibition of purified 20S proteasome activity (Fig. 1A). We suspected that higher concentrations of other proteasome inhibitors might be needed to reach their *in vivo* cellular target, the proteasome. If true, a specific authentic proteasome inhibitor should display differential potencies between purified proteasome and living cell proteasome activity. To test this idea, the effects of  $\beta$ -lactone, LLnL, and EGCG were measured on inhibition of the proteasomal chymotrypsin-like activity in intact Jurkat T cells. Fig. 3B demonstrates that  $\beta$ -lactone, LLnL, and EGCG at 10  $\mu$ M inhibited 20, 40, and 24% of the proteasomal chymotrypsin-like activity in living Jurkat cells, respectively, with the assay system used. The  $IC_{50}$  value of  $\beta$ -lactone to inhibit the chymotrypsin-like activity of a purified 20S proteasome was 0.6  $\mu$ M under our conditions (Fig. 1A) and 0.1–0.2  $\mu$ M under other conditions (34), and the  $IC_{50}$  value of LLnL to inhibit the 20S proteasome chymotrypsin-like activity was 0.14  $\mu$ M (37). Therefore, it appears that even for a specific proteasome inhibitor, higher concentrations are neces-



**FIG. 3. Inhibition of the proteasome activity by EGCG but not EGC in intact Jurkat cells.** Human Jurkat T cells were preincubated for 12 h with either the solvent (indicated by 0 or *cont.* for control) or EGCG, EGC,  $\beta$ -lactone, or LLnL at the indicated concentrations followed by an additional 2-h incubation with the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC (for the chymotrypsin-like activity of the proteasome) or Z-Gly-Gly-Arg-AMC (for the trypsin-like activity of the proteasome) (A and B). C, the medium was collected, and the free AMC groups were measured as described under "Experimental Procedures." Most of the data from A were derived from three independent experiments, except for EGCG and EGC at 10  $\mu$ M derived from six independent experiments to most accurately determine the validity of this important concentration.

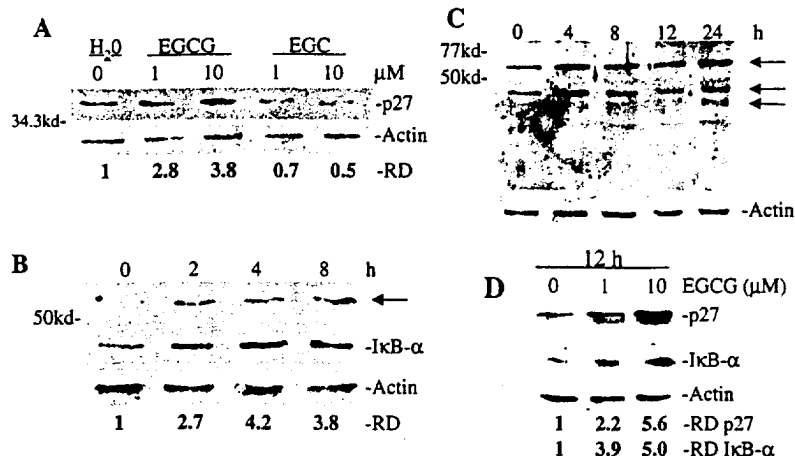
sary for the inhibition of the living cell proteasome activity. Because both  $\beta$ -lactone and EGCG showed greater potencies to purified 20S proteasome ( $IC_{50}$  values were 600 and 86 nM, respectively, Fig. 1A) than to intact cellular proteasome activity (20 and 24% inhibition at 10  $\mu$ M, respectively, Fig. 3B), EGCG seemed to be able to target, thereby inhibiting the proteasome in Jurkat T cells.

We also found that EGCG inhibited the proteasomal chymotrypsin-like activity in intact breast (MCF-7) and prostate (PC-3 and LNCaP) cancer cells (Fig. 4D and data not shown). However, EGCG did not inhibit the proteasomal trypsin-like activity in living Jurkat T cells (Fig. 3C). Taken together, our data suggest that EGCG but not EGC can selectively inhibit the chymotrypsin-like activity of purified 20S proteasome, 26S proteasome of tumor cell extracts, and 26S proteasome of living tumor cells.

To determine the molecular target(s) responsible for the cancer-preventative effects of green tea, one must adhere to the concentrations of the molecules, which are found physiologically in green tea drinkers. Previous studies indicate that EGCG or other catechins are present in low micromolar ranges



**FIG. 4. Accumulation of p27, I $\kappa$ B- $\alpha$ , and ubiquitinated proteins by EGCG.** A–C, lane 1, Jurkat T cells were treated with solvent, 1 or 10  $\mu$ M EGCG or EGC for 12 h (A), or 25  $\mu$ M EGCG for the indicated hours (B and C), or prostate cancer LNCaP cells were treated with solvent (lane 1 in A–D), 1 or 10  $\mu$ M EGCG, or EGC for 12 h (D), followed by a Western blot assay using specific antibodies to p27, I $\kappa$ B- $\alpha$ , actin, or ubiquitin. Molecular masses of I $\kappa$ B- $\alpha$  and actin are 40 and 43 kDa, respectively. The band of 56 kDa, indicated by an arrow in B might be an I $\kappa$ B- $\alpha$ -related protein. The bands indicated in C are ubiquitin-containing proteins. Relative density (RD) values are normalized ratios of the intensities of p27 or I $\kappa$ B- $\alpha$  band to the corresponding actin band.



in the plasma and saliva of human volunteers (3, 38) and in mice that had been fed with tea (38). Here we found that EGCG in low micromolar ranges acts as a potent proteasome inhibitor *in vitro* and *in vivo* (Figs. 1–3), indicating that EGCG at physiological levels could inhibit the proteasomal chymotrypsin-like activity in intact cancer cells and bring about the resultant tumor growth arrest (see below).

**Accumulation of the Proteasome Target Proteins p27<sup>Kip1</sup> and I $\kappa$ B- $\alpha$  in Tumor Cells Treated with EGCG**—To further confirm that EGCG inhibits the proteasome activity *in vivo*, Jurkat T cells were treated with various concentrations of EGCG or EGC for different hours followed by measuring levels of the cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> and I $\kappa$ B- $\alpha$ , two well known target proteins of the proteasome (29, 30). A 12-h treatment of Jurkat cells with 1  $\mu$ M EGCG increased p27 levels by ~3-fold (Fig. 4A, lane 2 versus lane 1), and the same treatment with 10  $\mu$ M EGCG increased p27 expression by ~4-fold (lane 3 versus lane 1). In contrast, EGC at the same concentrations had no such effect (Fig. 4A). EGCG treatment also increased I $\kappa$ B- $\alpha$  levels by 2.7-fold after a 2-h treatment and by ~4-fold after 4–8 h of treatment (Fig. 4B). A band of 56 kDa, detectable by the anti-I $\kappa$ B- $\alpha$  antibody used, was increased significantly during EGCG treatment (indicated by an arrow, Fig. 4B), suggesting that it might be an I $\kappa$ B- $\alpha$ -related protein.

Because most of the proteasome-mediated protein degradation pathways require ubiquitination (22), we expected that the inhibition of proteasome activity by EGCG should increase the levels of polyubiquitinated proteins. Indeed, when lysates of EGCG-treated Jurkat T cells were immunoblotted with an antiserum to ubiquitin, increased levels of several ubiquitinated proteins were detected (Fig. 4C).

To determine whether other cancer cell lines are also responsive to EGCG treatment, human prostate cancer LNCaP cells were treated with EGCG at 1 or 10  $\mu$ M for 12 h. Again, EGCG at 1  $\mu$ M increased the levels of p27 and I $\kappa$ B- $\alpha$  proteins by 2.2- and 3.9-fold, respectively, and EGCG at 10  $\mu$ M increased p27 and I $\kappa$ B- $\alpha$  expression by 5.6- and 5.0-fold, respectively, in these prostate cancer cells (Fig. 4D). Therefore, an accumulation of p27 and I $\kappa$ B- $\alpha$  proteins by EGCG treatment is time-dependent and concentration-dependent.

To rule out possible stimulatory effects of EGCG on the syntheses of p27 and I $\kappa$ B- $\alpha$  proteins, Jurkat T cells were pre-incubated with the protein synthesis inhibitor cycloheximide for 2 h followed by additional incubation with or without EGCG (in the presence of cycloheximide) to determine whether the stability of p27 and I $\kappa$ B- $\alpha$  proteins is increased by EGCG treatment. Incubation with cycloheximide alone significantly decreased the levels of both p27 and I $\kappa$ B- $\alpha$  proteins (Fig. 5, A and B, lanes 2 versus lanes 1). This decrease should be the result of

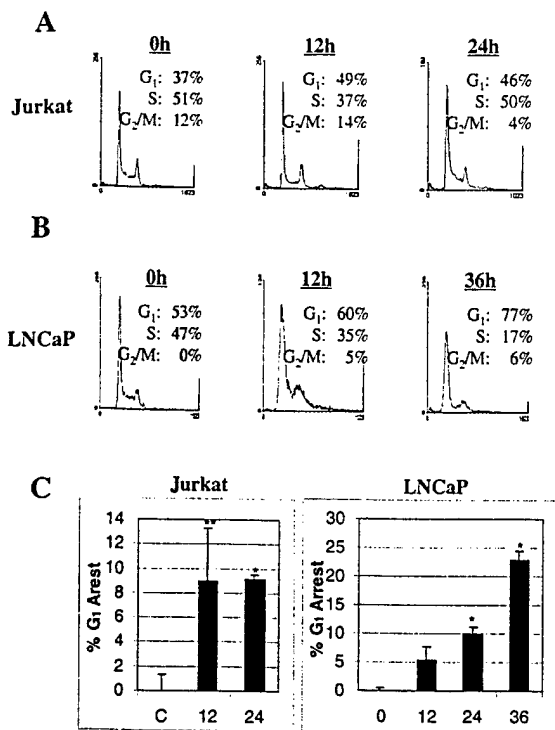
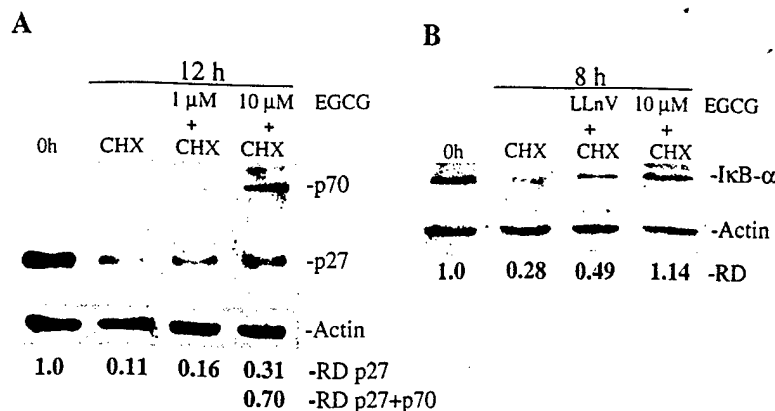
degradation of these proteins in the absence of new protein synthesis. When the cycloheximide-pretreated cells were coincubated with 10  $\mu$ M EGCG, the levels of p27 protein were increased by 3-fold with respect to cycloheximide treatment alone (Fig. 5A, lane 4 versus lane 2). This increase should be the result of the inhibition of p27 degradation by EGCG but not due to increased p27 synthesis because of the presence of cycloheximide. In addition, the appearance of a band of ~70 kDa (p70) was significantly increased by this treatment (Fig. 5A, lane 4 versus lane 2). The p70 may contain ubiquitinated p27, because a similar p70 containing ubiquitinated p27 was found in proteasome inhibitor-treated human osteosarcoma MG-63 cells (29) and breast cancer MDA-MB-231 cells (24). In fact, the sum of the levels of both p27 and p70 was increased by 7-fold in cells cotreated with cycloheximide and EGCG (Fig. 5A, lane 4 versus lane 2).

Coincubation of the cycloheximide-pretreated cells with 10  $\mu$ M EGCG also greatly increased the levels of I $\kappa$ B- $\alpha$  protein 4-fold higher than that of the cells treated with cycloheximide alone (Fig. 5B, lane 4 versus lane 2). The increase in I $\kappa$ B- $\alpha$  expression by EGCG was even greater than that by the proteasome inhibitor LLnV at the same concentration (4- versus 2-fold, Fig. 5B, lane 4 versus lane 3). Therefore, the inhibition of the proteasomal chymotrypsin-like activity in intact tumor cells (Fig. 3) correlates well with the accumulation of p27, I $\kappa$ B- $\alpha$ , and some ubiquitinated proteins (Figs. 4 and 5).

The following arguments support that inhibition of the proteasome activity by EGCG is responsible for the accumulation of p27 and I $\kappa$ B- $\alpha$  proteins in tumor (Figs. 4 and 5) and transformed (for review see Fig. 7) cells. First, as shown in Figs. 1–3, EGCG is a relatively potent specific proteasome inhibitor *in vitro* and *in vivo*. In addition, the accumulation of both p27 and I $\kappa$ B- $\alpha$  proteins was observed in an EGCG concentration-dependent (Figs. 4A and D and 5A) and time-dependent manner (Figs. 4B and 7). Furthermore, after EGCG treatment, the anti-p27 antibody detected a p70 band (Fig. 5A), which may contain ubiquitinated p27 (24, 29). Finally, the coincubation of cycloheximide-pretreated cells with EGCG demonstrated an almost complete inhibition of p27 and I $\kappa$ B- $\alpha$  protein degradation by EGCG (Fig. 5).

**EGCG Induces Tumor Cell Growth Arrest in G<sub>1</sub> Phase of the Cell Cycle**—It has been well documented that overexpression of either p27 (39, 40) or I $\kappa$ B- $\alpha$  (41, 42) suppresses the G<sub>1</sub>-to-S phase transition. If EGCG-accumulated p27 and I $\kappa$ B- $\alpha$  proteins (Fig. 4) were functional, the treated tumor cells should exhibit some growth arrest at G<sub>1</sub>. To test this possibility, Jurkat T or LNCaP cells were treated with EGCG under the similar conditions described in Fig. 4 and harvested for analysis of cell cycle distribution. A 12-h treatment of Jurkat T cells with 10

**FIG. 5. Accumulation of p27 and I $\kappa$ B- $\alpha$  proteins by EGCG in cycloheximide-pretreated cells.** Jurkat T cells were pretreated with 10  $\mu$ M cycloheximide for 2 h followed by coincubation with 1 or 10  $\mu$ M EGCG or 10  $\mu$ M LLnV for 8 or 12 h as indicated. This was followed by a Western blot assay using specific antibodies to p27, I $\kappa$ B- $\alpha$ , and actin. The p70 is a putative ubiquitinated p27-containing complex (29, 24). Relative density (RD) values are normalized ratios of intensities of p27 (or p27 plus p70) or I $\kappa$ B- $\alpha$  band to the corresponding actin band.



**FIG. 6. EGCG induces G<sub>1</sub> arrest in Jurkat T and LNCaP cancer cells.** Asynchronous (0 h) Jurkat (A) or LNCaP tumor cells (B) were treated with 10  $\mu$ M EGCG for indicated hours. At each time point, cells were harvested and analyzed by flow cytometry. Growth arrest is determined by the increase in the percentage of the G<sub>1</sub> population. C, statistical analysis. The results were derived from 3–5 independent experiments, and *p* values were calculated as indicated (\*, *p* < 0.01 as compared with respective 0 h; \*\*, *p* < 0.05 as compared with respective 0 h).

$\mu$ M EGCG increased G<sub>1</sub> population by 12% (Fig. 6A), consistent with the accumulation of p27 and I $\kappa$ B- $\alpha$  proteins under the same conditions (Fig. 4A). A 24-h treatment with EGCG remained an ~10% increase in G<sub>1</sub> population (Fig. 6A). The EGCG-induced Jurkat cell G<sub>1</sub> arrest was detected in several independent experiments that showed statistical significance (Fig. 6C, left).

Exposure of LNCaP prostate cancer cells to 10  $\mu$ M EGCG for 12 h initiated G<sub>1</sub> arrest by a 7% increase (Fig. 6B), which was correlated with p27 and I $\kappa$ B- $\alpha$  accumulation at this time (Fig. 4D). EGCG treatment of LNCaP cells for 24 and 36 h increased the G<sub>1</sub> population by 12 and 24%, respectively (Fig. 6B, and C, right). Again, the EGCG-mediated G<sub>1</sub> arrest of LNCaP prostate cancer cells was observed in multiple independent experiments (*p* < 0.01, Fig. 6C, right). These results support the functional significance of inhibition of the proteasome activity *in vivo* (Fig.

3) and the accumulation of p27 and I $\kappa$ B- $\alpha$  proteins by EGCG (Figs. 4 and 5). Our study is also consistent with previous reports that overexpression of p27 or I $\kappa$ B- $\alpha$  causes cell arrest in G<sub>1</sub> (39–42).

**Normal Human WI-38 Fibroblasts Are More Resistant to EGCG-induced p27 Accumulation and G<sub>1</sub> Arrest Than Their SV40-transformed Counterpart**—Previously, we reported that proteasome inhibitors selectively accumulated p27 protein and induced apoptosis in tumor and transformed abnormal human cells (24). To investigate whether EGCG has any differential effects on transformed and normal cells, the normal human fibroblast cell line WI-38 and its SV40-transformed derivative (VA-13) were treated with 10  $\mu$ M EGCG followed by the measurement of p27 and I $\kappa$ B- $\alpha$  protein levels and cell cycle distribution.

Similar to Jurkat T and LNCaP tumor cells (Fig. 4), the treatment of the transformed VA-13 cells with 10  $\mu$ M EGCG significantly increased p27 levels (Fig. 7A). A 12-h treatment with EGCG increased p27 expression by 2.8-fold; after 36 or 48 h, p27 levels were further increased by 7.6- and 9.2-fold, respectively (Fig. 7A). In contrast, the treatment of normal WI-38 cells with 10  $\mu$ M EGCG for up to 48 h did not increase p27 levels (Fig. 7A).

EGCG treatment of VA-13 cells also increased levels of I $\kappa$ B- $\alpha$  protein: 2.7-fold at 12 h, 8.9-fold at 36 h, and 4.2-fold at 48 h (Fig. 7B). Between 36 and 48 h, the levels of a p56 band associated with a decrease in I $\kappa$ B- $\alpha$  expression were increased in these transformed cells (Fig. 7B, indicated by an arrow), again suggesting that p56 is related to I $\kappa$ B- $\alpha$  (also see Fig. 4B). Although levels of I $\kappa$ B- $\alpha$  protein were low in the untreated normal WI-38 cells (0 h), a similar p56 protein was highly expressed (Fig. 7B). A 12-h treatment with EGCG increased the levels of I $\kappa$ B- $\alpha$  by 4.2-fold without affecting the p56 levels (Fig. 7B). EGCG treatment of WI-38 cells for 36–48 h did not further increase the levels of I $\kappa$ B- $\alpha$ , although under these conditions, the levels of p56 were decreased (Fig. 7B). As a control, actin levels were relatively unchanged during EGCG treatment in both VA-13 and WI-38 cells (Fig. 7C).

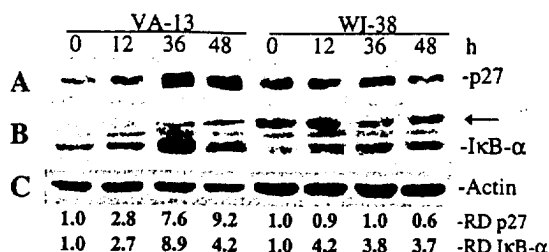
Correlated with the selective p27 accumulation in the transformed cells over normal WI-38 cells by EGCG (Fig. 7A), VA-13 cells were found to be more sensitive to EGCG-induced G<sub>1</sub> arrest than WI-38 cells. After a 12-h treatment with EGCG, the G<sub>1</sub> population of VA-13 cells was increased by 22% (Fig. 8, A and C). In contrast, no apparent G<sub>1</sub> arrest was observed in WI-38 cells under this condition (Fig. 8, B and C). At 24 h, the G<sub>1</sub> population of the transformed cells was further increased (by ~25%); the WI-38 G<sub>1</sub> population began to increase (by <5%) (Fig. 8C). After a 36-h treatment, the VA-13 G<sub>1</sub> population continued to increase (by 33%, Fig. 8, A and C); only at this time, a 16% increase in the G<sub>1</sub> population of WI-38 cells was also detected (Fig. 8, B and C). The results from several inde-

pendent experiments confirmed that the transformed VA-13 cells were more sensitive to EGCG-induced  $G_1$  arrest than the normal WI-38 cells (Fig. 8C). It appears that the delayed EGCG-induced  $G_1$  arrest in WI-38 cells is associated with the lack of p27 accumulation and the partial induction of I $\kappa$ B- $\alpha$  expression in these normal human fibroblasts (Fig. 7, A and B). Previously, other researchers reported that EGCG has a pronounced growth inhibitory effect on cancerous but not on their

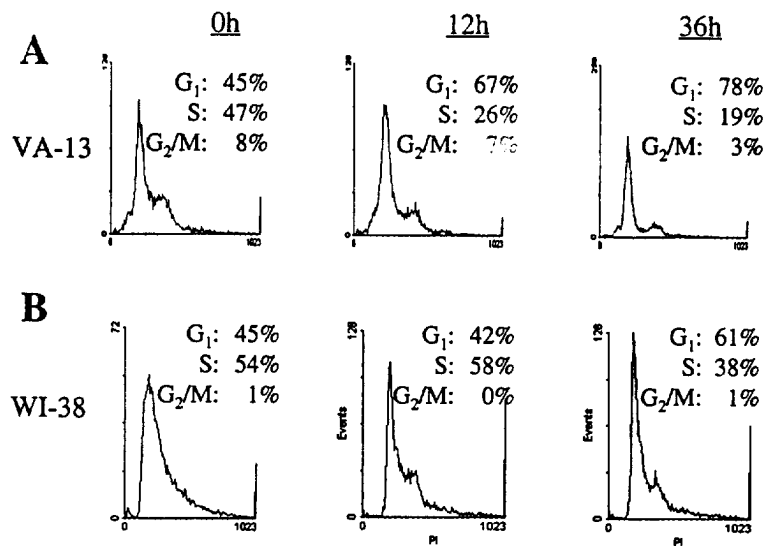
normal counterparts (43). Our study has extended their observation by providing a molecular mechanism for such a selectivity of EGCG.

**HPLC Analysis of EGCG After Reaction with Purified Proteasome**—The ester bond carbon in  $\beta$ -lactone can be attacked by the strong nucleophilic hydroxyl group of N-terminal threonine residue of the proteasome, forming a covalent complex (32, 33). We hypothesized that the ester bond of EGCG would be attacked by the N-terminal threonine residue of the proteasome, forming a covalent (or tight) EGCG-proteasome complex, thus inactivating the proteasome. If so, EGCG should be quickly lost, associated with production of no (or little) cleavage products of EGCG such as gallic acid and EGC (for review see Fig. 1A).

To test this hypothesis, a highly purified EGCG (for review see Fig. 9D) was incubated with purified 20S proteasome for various hours followed by HPLC analysis. After a 2-h incubation, a gallic acid-like peak (retention time 4.78) associated with a 40% decrease in the level of EGCG was detected in the HPLC chromatogram whose level was corresponded to a concentration of <5% EGCG (Fig. 9, A and C and Fig. 10). The gallic acid-like peak was not produced from EGCG in the absence of the proteasome (Fig. 9D). The incubation of EGCG with purified proteasome for 4 h resulted in the disappearance



**FIG. 7. Preferable accumulation of p27 protein by EGCG in the transformed fibroblasts over the normal human fibroblasts.** Normal (WI-38) and SV40-transformed (VA-13) human fibroblasts were treated with 10  $\mu$ M EGCG for the indicated hours followed by a Western blot assay using specific antibodies to p27 (A), I $\kappa$ B- $\alpha$  (B), and actin (C) as described in the legend of Fig. 4. The band of 56 kDa, indicated by an arrow in B, might be an I $\kappa$ B- $\alpha$ -related protein.



**FIG. 8. Differential sensitivity of the transformed fibroblasts and the normal human fibroblasts to EGCG-induced  $G_1$  arrest.** Normal (WI-38) (B) and SV40-transformed (VA-13) (A) human fibroblasts were treated with 10  $\mu$ M EGCG for the indicated hours followed by flow cytometry analysis. Growth arrest is determined by the increase in the percentage of  $G_1$  population. C, statistical analysis. The results were derived from five independent experiments, and  $p$  values were calculated as indicated (\*,  $p < 0.01$  as compared with respective 0 h; \*\*,  $p < 0.05$  as compared with respective 0 h).

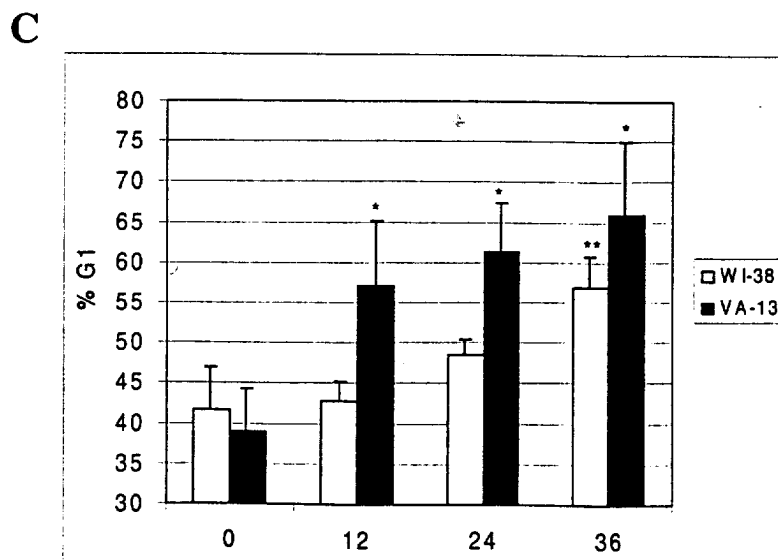


FIG. 9. HPLC chromatograms of EGCG after incubation with purified 20S proteasome. EGCG or EGC was incubated with either a purified 20S proteasome (Pr.) or the buffer (buff.) for the indicated hours followed by HPLC analysis. The retention times of reaction products were confirmed by using tea polyphenol standards (Std.). For details, see under "Experimental Procedures."

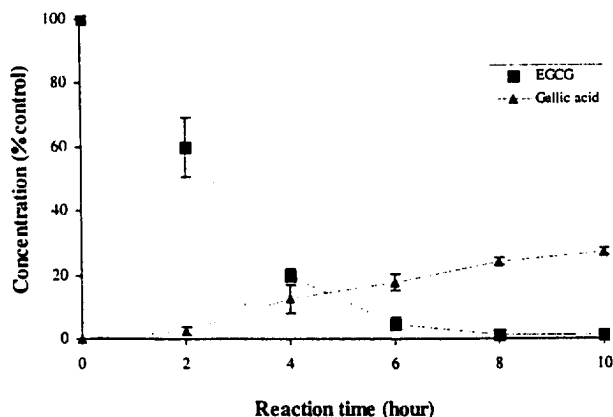
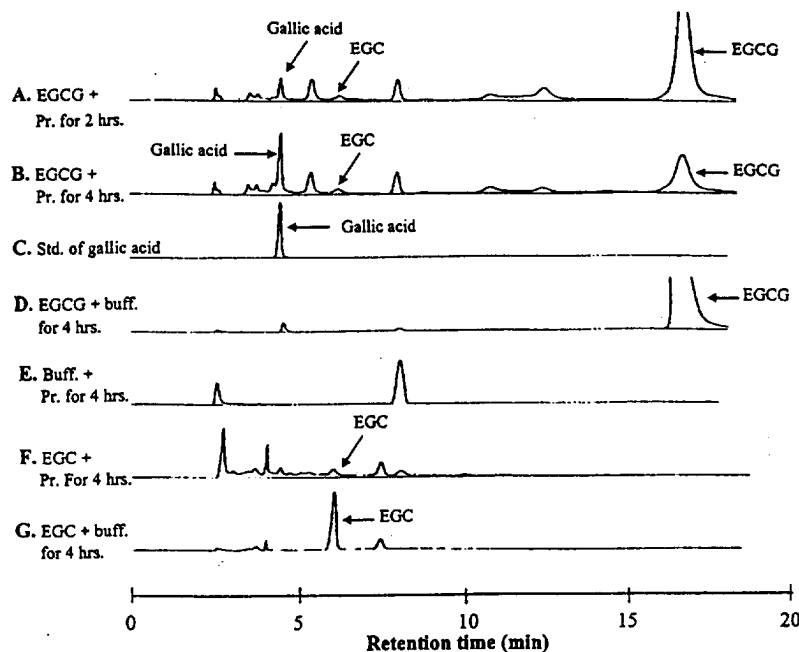


FIG. 10. The disappearance of EGCG occurs prior to the appearance of gallic acid-like product after incubation with purified 20S proteasome. EGCG was incubated with purified 20S proteasome for indicated hours followed by HPLC analysis. The levels of EGCG and gallic acid-like product were measured and plotted.

of an ~80% EGCG, associated with an increase in the level of the gallic acid-like peak that is equivalent to a concentration of 13% EGCG (Fig. 9, B versus A and Fig. 10). After a 6–10 h incubation with the proteasome, >95% EGCG disappeared, whereas the level of the gallic acid-like product linearly increased to a level equivalent to 20–25% EGCG (Fig. 10).

The incubation of EGCG with the 20S proteasome also resulted in the appearance of an EGC-like product (retention time 6.62 min), although its level was very low (Fig. 9, A and B versus G), suggesting that the produced EGC could be further degraded by the proteasome. Indeed, purified EGC was degraded almost completely by purified 20S proteasome (Fig. 9, F versus G). Several unknown products of EGCG, including one with a retention time of 5.75 min between gallic acid and EGC peaks, were observed (Fig. 9, A and B). Another unknown peak at a retention time of ~8 min resulted from the mixture of the buffer and purified proteasome (compare Fig. 9 A and B with E). Taken together, the disappearance of most of the free EGCG prior to the appearance of low levels of gallic acid-like product suggests that EGCG might form a tight complex with

the proteasome. This hypothesis is consistent with the observed potency of EGCG as a proteasome inhibitor *in vitro* (Figs. 1 and 2) and *in vivo* (Figs. 3–5 and 7). The HPLC data also suggest that the proteasome-bound EGCG could be slowly cleaved at one or more places including the ester bond, which leads to the production of gallic acid, EGC, and other products. Finally, a complete cleavage of EGC by the purified proteasome (Fig. 9F) is consistent with failure of EGC to inhibit the purified proteasome activity (Fig. 1A) and the proteasome activity in Jurkat cell extracts (Fig. 2A) and intact Jurkat T cells (Figs. 3A and 4A).

Based on our current study, we propose the following molecular mechanisms by which EGCG inhibits the proteasome. The two nucleophilic electrons located on the N-terminal threonine hydroxyl group of the proteasome subunit X (32–34) could attack the ester bond carbon of EGCG after binding to the proteasome active site. A tight EGCG-proteasome complex could be generated, thereby inactivating the proteasome. This complex would slowly disassociate to free EGC and gallic acid. Further studies are needed to understand the nature of ester bond-containing polyphenols as a potent proteasome inhibitor.

In summary, for many years it has been shown through epidemiological studies that green tea is a cancer-preventative agent (1–4). It has also been shown that the proteasome plays an important role in the development and progression of cancer (22, 23). Our study has demonstrated for the first time that the compounds found in tea and in the bodies of green tea drinkers can inhibit the proteasome at or near physiological concentrations. Our results also indicate that the inhibition of the proteasome activity by EGCG can selectively control the growth of tumor and transformed cells. We suggest that the cancer-preventative properties of green tea could be attributed, at least in part, to its ability to inhibit the proteasome activity. Our finding along with the low toxicity of EGCG also implicates the role of tea in a potential clinical therapy in combination with current anticancer drugs.

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# Tannic Acid Potently Inhibits Tumor Cell Proteasome Activity, Increases p27 and Bax Expression, and Induces G<sub>1</sub> Arrest and Apoptosis<sup>1</sup>

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## Abstract

Animal studies have demonstrated that a dietary polyphenol known as tannic acid (TA) exhibits anticarcinogenic activity in chemically induced cancers, although the involved molecular target remains unknown. In addition, proteasome inhibitors have been shown to suppress human tumor growth in nude mice. Most recently, we have reported that ester-bond-containing tea polyphenols are potent proteasome inhibitors *in vitro* and *in vivo*. We have hypothesized that TA, which contains multiple similar gallate moieties linked by ester bonds, should inhibit the proteasome activity. Here, we report that indeed TA potently and specifically inhibits the chymotrypsin-like activity of purified 20S proteasome (IC<sub>50</sub> = 0.06 µg/ml), 26S proteasome of Jurkat T-cell extracts, and 26S proteasome of living Jurkat cells. Inhibition of the proteasome by TA in Jurkat cells results in accumulation of two natural proteasome substrates, the cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> and the proapoptotic protein Bax, followed by growth arrest in G<sub>1</sub> and induction of apoptotic cell death. Our present study suggests that TA targets and inhibits the proteasome in tumor cells, which may contribute to the previously observed anticarcinogenic activity of TA.

## Introduction

Tannins are plant-derived polyphenolic compounds with molecular weights of 500–3000 Da, which can be classified into two groups, hydrolysable and condensed tannins (1–3). The hydrolysable tannins, commonly called TA,<sup>3</sup> contain either gallotannins or ellagitannins. On hydrolysis, gallotannins yield glu-

cose and gallic acid (Fig. 1). TA is widely found in food plants and broadly applied to various industrial food additives (1–3). It is estimated that a person on a balanced diet ingests 1 gm of TA every day in the United States (4).

Recently, it has been shown that TA exerts cancer chemopreventative activity in various animal models (3), *e.g.*, TA was able to suppress skin tumor promotion induced by UV-B radiation in hairless mice by ≤70% (5). In addition, TA dietary intake in low doses can exert a strong dose-dependent chemopreventative activity against spontaneous liver tumor development in C3H male mice by ≤87% (6). Furthermore, it has been shown that TA increased survival rate of BALB/c mice bearing syngeneic tumors by ≤30% (7).

Although the molecular mechanisms responsible for the cancer chemopreventative activity of TA remain unknown, *in vitro* studies have suggested a contribution of the apoptosis-inducing activity of TA. Along this line, TA has been shown to induce either growth arrest (8) or apoptotic death (9). Furthermore, TA induced apoptosis preferably in human oral squamous cell carcinoma and salivary gland tumor cell lines than in normal human gingival fibroblasts, whereas gallic acid, a component unit of TA, showed much weaker selective cytotoxicity (10). However, a conclusive mechanistic target protein responsible for the anticancer property of TA has not been identified.

The 20S proteasome, a multicatalytic complex (700 kDa), constitutes the catalytic component of the ubiquitous proteolytic machinery of the 26S proteasome (11–14). The ubiquitin-proteasome system plays a critical role in the specific degradation of cellular proteins, and two important functions of the proteasome are to promote tumor cell proliferation and to protect tumor cells against apoptosis (11–14). It has been shown that the chymotrypsin-like, but not trypsin-like, activity of the proteasome is associated with tumor cell survival (15, 16). Several cell proliferation and cell death regulators have been identified as targets of the ubiquitin/proteasome-mediated degradation pathway, including p53 (17), pRb (18), p21 (19), p27<sup>Kip1</sup> (20), IκB-α (21), and Bax (22).

Most recently, we have reported that ester bond-containing tea polyphenols, such as (-)-epigallocatechin-3-gallate, potently and specifically inhibited the chymotrypsin-like activity of the proteasome *in vitro* (IC<sub>50</sub> 86–194 nM) and *in vivo* (1–10 µM) at the concentrations found in the serum of green tea drinkers (23). Because TA contains several ester bonds (Ref. 2; Fig. 1), we have hypothesized that TA could also inhibit proteasomal activity. Here, we report that ester-bond containing TA potently and selectively inhibits the chymotrypsin-like activity in purified 20S proteasome, 26S proteasome of Jurkat T-cell extracts, and 26S proteasome in intact Jurkat cells. Furthermore, inhibition of the proteasome by TA in Jurkat T cells is associated with accumulation of the cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> and proapoptotic protein Bax and is accompanied by induction of G<sub>1</sub> arrest and apoptosis.

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<sup>3</sup> The abbreviations used are: TA, tannic acid; AMC, 7-amido-4-methyl-coumarin; PARP, poly(ADP-Ribose) polymerase.

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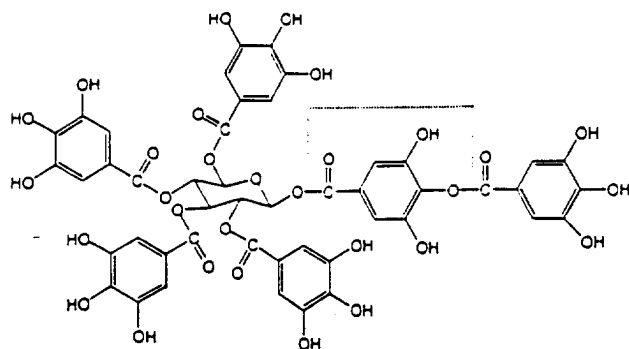


Fig. 1. Structure of gallotannin (TA).

## Materials and Methods

**Materials.** Highly purified TA (gallotannin; ACS Reagent) and D-(+)-glucose (>99.5%) were purchased from Sigma Chemical Co. (St. Louis, MO) and used directly without additional purification. Purified 20S proteasome (*Methanosarcina thermophila*, Recombinant, *Escherichia coli*) and purified calpain I (Human Erythrocytes) were purchased from Calbiochem (La Jolla, CA). Fluorogenic peptide substrates Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) and Suc-Leu-Tyr-AMC (for the calpain I activity) were obtained from Calbiochem, and Z-Gly-Gly-Arg-AMC (for the proteasomal trypsin-like activity) was obtained from Bachem (King of Prussia, PA). The specific calpain inhibitor calpeptin was obtained from Calbiochem. Monoclonal antibody to p27<sup>Kip</sup> was purchased from PharMingen (San Diego, CA), rabbit polyclonal antibody to human PARP was obtained from Boehringer Mannheim, and human Bax (clone N-20) and actin (clone C11) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Cell Culture and Cell Extract Preparation.** Human Jurkat T cells were cultured in RPMI 1640, supplemented with 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained in a 5% CO<sub>2</sub> atmosphere at 37°C. A whole cell extract was prepared as described previously (15). Briefly, cells were harvested, washed with PBS twice, and homogenized in a lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol] for 30 min at 4°C. After that, the lysates were centrifuged, and the supernatants were collected as whole cell extracts.

**Inhibition of Purified 20S Proteasome Activity by TA.** The chymotrypsin-like activity of purified 20S proteasome was measured as follows. Briefly, 0.5 µg of purified 20S proteasome was incubated with 20 µM fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity), for 30 min at 37°C in 100 µl of assay buffer [20 mM Tris-HCl (pH 8.0)] with or without TA. After incubation, the reaction mixture was diluted to 200 µl with the assay buffer, followed by measurement of the hydrolyzed AMC groups using a VersaFluor Fluorometer with an excitation filter of 380 nm and an emission filter of 460 nm (Bio-Rad).

**Inhibition of the Proteasome Activity in Whole Cell Extracts by TA.** Jurkat cell extract (6 µg) was incubated for 90 min at 37°C with 20 µM fluorogenic peptide substrate (Suc-Leu-Leu-Val-Tyr-AMC or Z-Gly-Gly-Arg-AMC, for chymotrypsin-like or trypsin-like activities of the proteasome, respectively) in 100 µl of the assay buffer in the presence or absence

of TA. The hydrolyzed AMCs were quantified as described above.

**Inhibition of the Proteasome Activity in Intact Jurkat T Cells by TA.** To measure inhibition of the proteasome activity in living tumor cells, Jurkat T cells ( $1 \times 10^5$  cells/ml/well) were cultured in a 24-well plate. These cells were first incubated for 12 h with various concentrations of TA, followed by an additional 2-h incubation with the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC. After that, cell medium (200 µl/sample) was collected and used for measurement of free AMCs.

**Calpain I Activity Assay.** To measure calpain I activity, 3 µg of purified calpain I was incubated with 40 µM fluorogenic peptide calpain substrate, Suc-Leu-Tyr-AMC, for 30 min at 37°C in 100 µl of assay buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 5 mM CaCl<sub>2</sub>, and 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, with or without TA or the specific calpain inhibitor calpeptin (24). After incubation, the reaction mixture was diluted to 200 µl with the assay buffer, and the hydrolyzed AMCs were quantified as described above.

**Western Blot Analysis.** The enhanced chemiluminescence Western Blot analysis was performed using specific antibodies to p27<sup>Kip</sup>, Bax, PARP, or actin, as described previously (15). Briefly, Jurkat T cells were treated with TA for indicated hours (see figure legends), harvested, and lysated in the lysis buffer. Cell lysates (70 µg) were separated by an SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane, followed by enhanced chemiluminescence Western blotting.

**Flow Cytometry.** Cell cycle analysis based on DNA content was performed as follows. At each time point, cells were harvested, counted, and washed twice with PBS. Cells ( $5 \times 10^6$ ) were suspended in 0.5 ml of PBS, fixed in 5 ml of 80% ethanol for overnight at -20°C, centrifuged, resuspended again in 1 ml of propidium iodide staining solution (50 µg of propidium iodide, 100 units of RNase A, and 1 mg of glucose per ml PBS), and incubated at room temperature for 30 min. The cells were then analyzed with FACScan (Becton Dickinson Immunocytometry Systems, CA) and ModFit LT cell cycle analysis software (Verity Software, Topsham, ME). The cell cycle distribution is presented as the percentage of cells containing G<sub>1</sub>, S, G<sub>2</sub>, and M DNA content as judged by propidium iodide staining. Cell death-associated DNA degradation is determined as the percentage of cells containing <G<sub>1</sub> DNA content (Pre-G<sub>1</sub>).

## Results

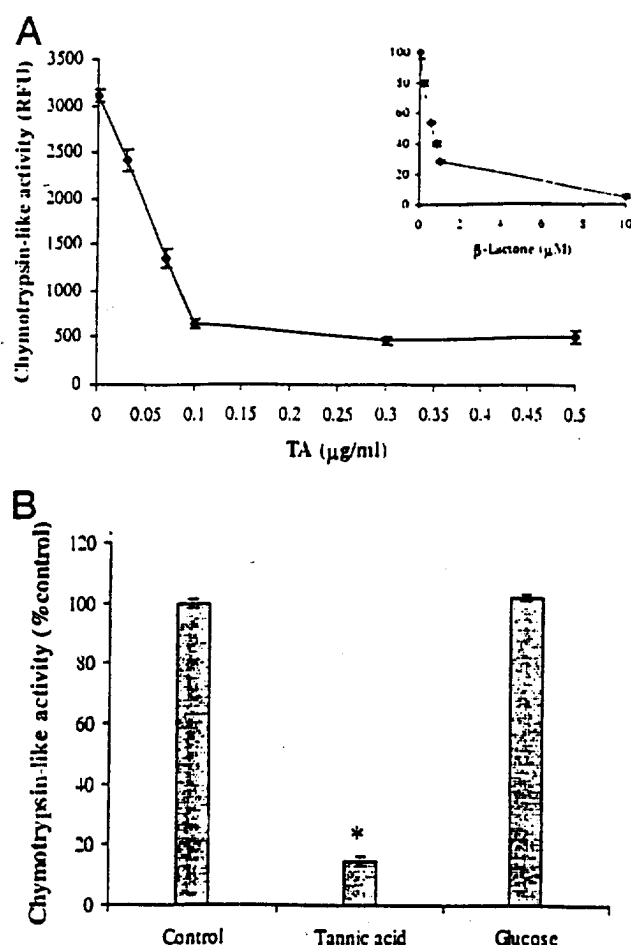
**TA Potently Inhibits the Chymotrypsin-like Activity of Purified 20S Proteasome.** Gallotannin is composed of a D-glucose as a core that is linked by six to nine gallic groups through ester bonds (Fig. 1). Because ester bond-containing tea polyphenols are potent proteasome inhibitors (23), we hypothesized that TA would inhibit the proteasome activity. To test this hypothesis, we performed a cell-free proteasome activity assay with or without TA. The result in Fig. 2A demonstrates that TA potently inhibited the chymotrypsin-like activity of purified 20S proteasome with an IC<sub>50</sub> value of 0.06 µg/ml (Fig. 2A). The shape of the inhibition curve of TA was similar to that of the specific proteasome inhibitor clastolactacystin β-lactone (Fig. 2A, insert; Ref. 23), consistent with the conclusion that TA acts as a proteasome inhibitor.

We next determined whether an individual moiety of TA, such as D-glucose or gallic acid, has any proteasome inhibitory

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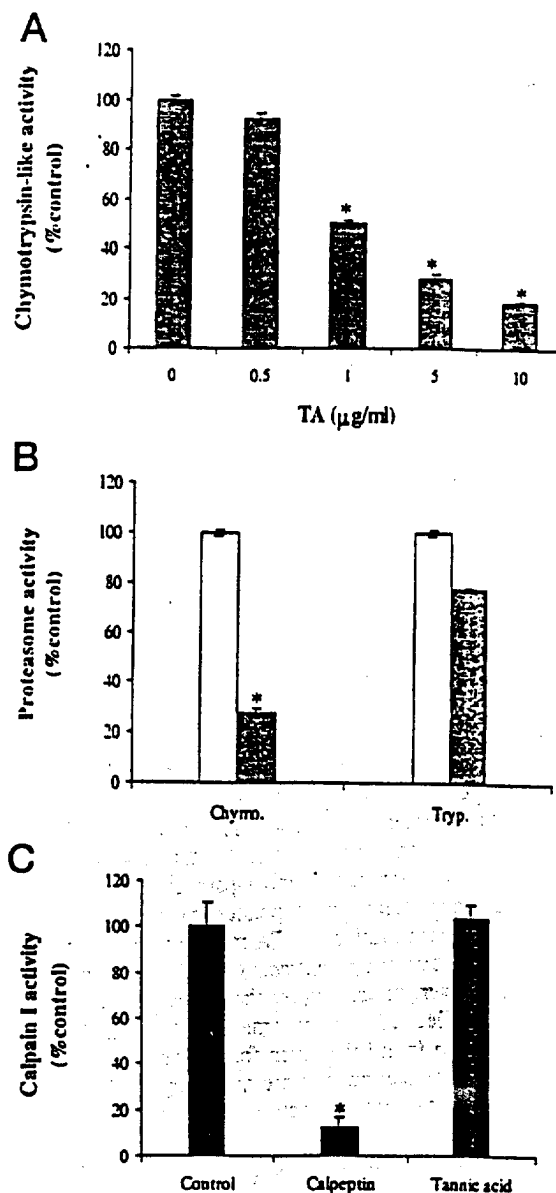


**Fig. 2.** Inhibition of the purified 20S proteasome activity *in vitro* by TA. In A, 0.5 μg of purified 20S proteasome was incubated with 20 μM Suc-Leu-Leu-Val-Tyr-AMC with TA at various concentrations. Inhibitory activity of TA toward the chymotrypsin-like activity of the purified 20S proteasome was measured as described in "Materials and Methods." *Insert*, concentration-dependent inhibition of the chymotrypsin-like activity of the purified 20S proteasome by β-lactone (23). B, similar to A, effects of TA (0.3 μg/ml) and D-glucose (180 μg/ml) on the chymotrypsin-like activity of the purified 20S proteasome were measured. The values of the error bars are the mean ± SD of three independent experiments. \**P* < 0.05, compared with the control.

activity. We found that D-glucose at a very high concentration (180 μg/ml) did not affect the chymotrypsin-like activity of purified 20S proteasome. As a comparison, TA at a 600-fold lower concentration (0.3 μg/ml) inhibited >80% of the 20S proteasomal activity (Fig. 2B). In addition, gallate also failed to inhibit the proteasomal activity (data not shown and Ref. 23). These results indicate that the ester bonds of TA play an essential role in inhibition of the proteasomal chymotrypsin-like activity.

**TA Inhibits the Proteasomal Chymotrypsin-like Activity in Tumor Cell Extracts.** We then tested if TA could inhibit the 26S proteasome activity in a tumor cell extract. Protein extract was prepared from exponentially growing human Jurkat T cells and used in the cell-free proteasome activity assay. We found that TA also potently inhibited the proteasomal chymotrypsin-like activity in Jurkat T-cell extract in a concentration-dependent manner: ~50% inhibition at 1 μg/ml and ~80% at 10 μg/ml (Fig. 3A).

To study the specificity of TA-mediated inhibition, its effects on the proteasomal trypsin-like and calpain protease activities were then investigated. In Jurkat T-cell extract,



**Fig. 3.** Selective inhibition of the proteasomal chymotrypsin-like activity by TA. In A, Jurkat cell extract (6 μg) was incubated with 20 μM Suc-Leu-Leu-Val-Tyr-AMC (for the chymotrypsin-like activity) and TA at indicated concentrations. In B, Jurkat cell extract (6 μg) was incubated with 20 μM Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) or Z-Gly-Gly-Arg-AMC (for the proteasomal trypsin-like activity) in the presence of 5 μg/ml TA. In C, purified calpain I (3 μg) protein was incubated with 40 μM fluorogenic peptide substrate, Suc-Leu-Tyr-AMC, with the vehicle DMSO (Control), TA (5 μg/ml), or the specific calpain inhibitor calpeptin (0.18 μg/ml). After incubation, the hydrolyzed AMC were quantified as described in Fig. 2. The values of the error bars are the mean ± SD of three independent experiments. \**P* < 0.05, compared with the control.

23% of the trypsin-like activity of the proteasome, in contrast to a 73% inhibition of the chymotrypsin-like activity in a Jurkat T-cell extract (Fig. 3B). In addition, TA at 5 μg/ml had no inhibitory effects on the purified calpain I activity (Fig. 3C), although at 0.06 μg/ml, TA inhibited 50% of the chymotrypsin-like activity of purified 20S proteasome (Fig. 2A). As a positive control, the specific calpain inhibitor calpeptin (24) at a 28-fold lower concentration (0.18 μg/ml) inhibited >85% of the purified calpain I activity (Fig. 3C). These data suggest that TA preferably inhibits the chymotrypsin-like activity of the protea-



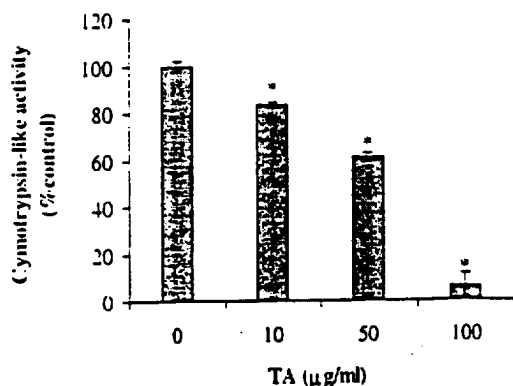


Fig. 4. Inhibition of the chymotrypsin-like activity by TA in intact Jurkat T cells. Intact Jurkat T cells ( $1 \times 10^5$  cells/ml/well) were preincubated for 12 h with various concentrations of TA, followed by an additional 2-h incubation with the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the chymotrypsin-like activity). The medium was collected, and the free AMC groups were measured as described in "Materials and Methods." The values of the error bars are the mean  $\pm$  SD of three independent experiments. \* $P < 0.05$ , compared with the control.

TA Inhibits the Proteasomal Chymotrypsin-like Activity in Intact Jurkat T Cells. To determine whether TA could also inhibit the living cell proteasomal chymotrypsin-like activity, Jurkat T cells were first incubated with various concentrations of TA, followed by an additional incubation with the fluorogenic proteasome peptide substrate. After that, cell medium was collected for measurement of hydrolyzed products (free AMCs). By performing this assay, we found that TA significantly inhibited the proteasomal chymotrypsin-like activity in intact Jurkat cells in a concentration-dependent manner (Fig. 4). We noticed that the concentrations of TA needed to inhibit the proteasome activity in Jurkat cell extracts (Fig. 3A), and intact Jurkat cells (Fig. 4) were much higher than were needed for inhibition of the purified 20S proteasome activity (Fig. 2A). However, we have also found that even for a specific proteasome inhibitor, higher concentrations are necessary for inhibition of the living cell proteasome activity (Ref. 23 and see "Discussion").

Accumulation of the Proteasome Target Proteins p27<sup>Kip1</sup> and Bax in Jurkat T Cells Treated with TA. If TA inhibits the proteasome activity *in vivo*, we would expect to see an increase in levels of proteasome target proteins. To investigate this possibility, Jurkat T cells were treated with TA for  $\leq 24$  h, followed by measuring levels of the cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> and the proapoptotic protein Bax, two well-known target proteins of the proteasome (20, 22). Treatment of TA at 50  $\mu$ g/ml increased p27 levels by 2-fold at 4 h and by 3-fold at 12 and 24 h (Fig. 5A). When TA was used at 100  $\mu$ g/ml, much greater effect was observed; p27 was increased by 8- to 11-fold (Fig. 5B). Furthermore, TA at 50 (Fig. 5C) or 100  $\mu$ g/ml (data not shown) also induced Bax expression by 3- to 7-fold. Levels of actin were found to be relatively unchanged during the TA treatment, which was used as a loading control (Fig. 5, A-C).

TA Induces G<sub>1</sub> Arrest and Apoptotic Cell Death. It has been documented that p27 acts as an inhibitor of the G<sub>1</sub> to S phase transition (25, 26). If p27 protein accumulated by TA (Fig. 5, A and B) was functional, the TA-treated tumor cells should exhibit some growth arrest at G<sub>1</sub>. To test this possibility, Jurkat T cells were treated with TA under the same conditions described in Fig. 5 and harvested for analysis of cell cycle distribution.

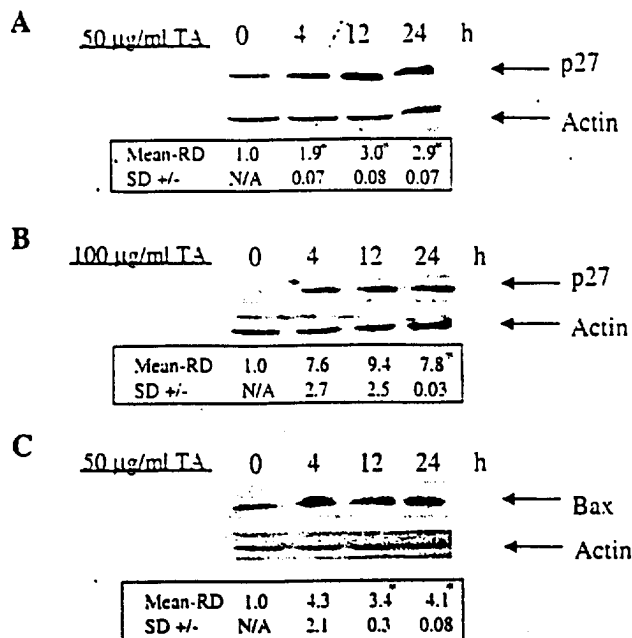


Fig. 5. Accumulation of p27 and Bax proteins in Jurkat T cells treated with TA. Jurkat T cells were treated with 50 (A and C) or 100  $\mu$ g/ml (B) of TA for the indicated hours, followed by Western blot assay using specific antibodies to p27, Bax (MW 21 kDa), or actin (43 kDa), respectively. RD (relative density) values are normalized ratios of the intensities of p27 or Bax band to the corresponding actin band. The values are the mean  $\pm$  SD of four independent experiments. \* $P < 0.05$ , compared with the control.

Compared with the vehicle-treated cells (Control), treatment with TA at 50  $\mu$ g/ml increased G<sub>1</sub> population by 5% at 4 h and 15% at 12 h (Fig. 6) before induction of cell death (see below).

Bax has been shown to be an apoptotic cell death promoter (27, 28). We then investigated whether cell death had occurred in TA-treated Jurkat T cells, in association with the increased Bax protein levels (Fig. 5C). The first cell death index used was the cell population with  $< G_1$  DNA content (indicated by pre-G<sub>1</sub>), which measures cell death-associated DNA degradation and can be determined by flow cytometry (15). Another cell death index was the apoptosis-specific cleavage of PARP, which is carried out by activated caspase-3 or -7 and can be measured by Western blotting (27, 28, 22).

Treatment of Jurkat cells with TA at 50  $\mu$ g/ml for 24 h significantly increased cell death, as judged by a 15% increase in the pre-G<sub>1</sub> cell population (Fig. 6). The p85 PARP cleavage fragment was also detected under the same experimental condition (Fig. 7A, Lane 4), suggesting induction of apoptotic cell death. At 48 h, cell death was additionally increased, as shown by the 25% increase in the pre-G<sub>1</sub> cell population (Fig. 6) and additional increase in the level of p85/PARP cleavage fragment (Fig. 7A, Lane 5).

Treatment with TA at 100  $\mu$ g/ml had greater apoptosis-inducing effect than at 50  $\mu$ g/ml, because PARP cleavage occurred earlier (at 12 h), and higher levels of p85 PARP cleavage fragment were observed at a fixed time point (12, 24, or 48 h; Fig. 7, B versus A). In addition, when Jurkat T cells were treated with various concentrations of TA (10–100  $\mu$ g/ml) for 24 h, the pre-G<sub>1</sub> cell population was increased in a concentration-dependent manner. Therefore, TA-induced cell death was time- and concentration-dependent.

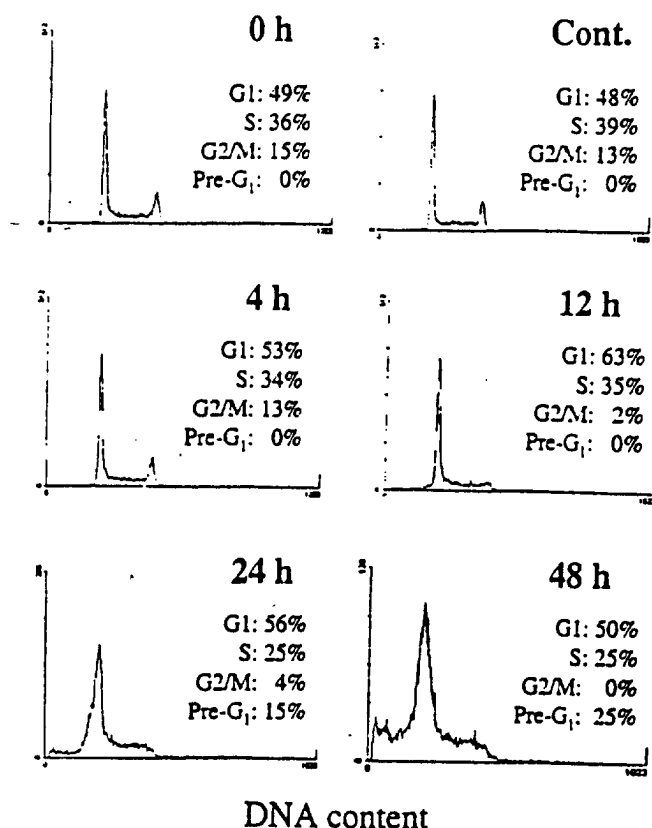


Fig. 6. Induction of G<sub>1</sub> arrest and cell death by TA in Jurkat T cells. Exponentially grown Jurkat T cells (0 h) were treated with 50 µg/ml TA for indicated hours. Cont., control cells treated with the vehicle (H<sub>2</sub>O) for 4, 12, 24, or 48 h. All of the control-treated cells exhibited similar cell cycle distribution. At each time point, cells were harvested and analyzed by flow cytometry. Growth arrest is determined by the increase in the percentage of G<sub>1</sub> population, and cell death-associated DNA degradation is measured by the increase in the percentage of cell population with <G<sub>1</sub> DNA content (Pre-G<sub>1</sub>). Similar results were observed in three independent experiments.

## Discussion

Recent animal studies have suggested that TA has a cancer-preventative activity (3, 5–7). Cell culture studies also indicate that TA can induce either growth arrest (8) or apoptosis (9, 10). However, the involved molecular target(s) have not been identified. In the current study, we demonstrated that TA was a potent inhibitor of the proteasomal chymotrypsin-like activity both *in vitro* and *in vivo*. Inhibition of the proteasome activity by TA in intact Jurkat T cells resulted in accumulation of p27 and Bax, associated with G<sub>1</sub> arrest and apoptosis. This finding is consistent with previous reports that show inhibition of the chymotrypsin-like, but not trypsin-like, activity of the proteasome by a specific inhibitor was sufficient to induce either tumor cell growth arrest or apoptosis (15, 16).

It has been shown that the ester bond carbon of  $\beta$ -lactone is responsible for potently and specifically inhibiting the proteasome (29). Our results suggest that ester bonds present in TA are also responsible for its proteasome inhibitory potency. Indeed, each moiety itself of TA, D-glucose, or gallic acid did not inhibit the proteasome activity *in vitro* (Fig. 2B and Ref. 23). In addition to the inhibitory potency of TA against the proteasomal chymotrypsin-like activity, the inhibitory specificity of TA was also investigated by testing its effects on other proteasomal or protease activities. TA did not inhibit the activity of purified calpain I, in contrast to being a potent inhibitor of the chymotrypsin-like activity of purified 20S proteasome (Fig. 3C versus

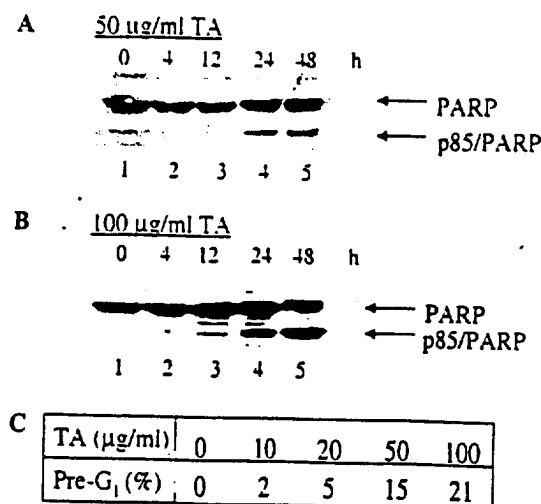


Fig. 7. TA induces Jurkat cell apoptosis in a concentration-dependent manner. Jurkat T cells were treated with 50 (A) or 100 µg/ml (B) TA for the indicated hours, followed by Western blot assay using a specific PARP antibody. The intact PARP (116 kDa) and a PARP cleavage fragment (85 kDa) are indicated. In C, Jurkat T cells were treated for 24 h with TA at indicated concentrations, followed by flow cytometry. The cell populations with <G<sub>1</sub> DNA content (Pre-G<sub>1</sub>) are shown. Similar results were observed in four independent experiments.

24). TA was also much less potent against the proteasomal trypsin-like activity than against the chymotrypsin-like activity in tumor cell extracts (Fig. 3B). These results at least suggest that TA preferably inhibits the chymotrypsin-like activity of the proteasome.

When we compared the *in vitro* and *in vivo* potencies of TA, we noted that that ~0.1 µg/ml TA was needed to inhibit ~85% of the chymotrypsin-like activity of purified 20S proteasome (Fig. 2A), whereas 10 µg/ml TA was needed for 80% inhibition of the chymotrypsin-like activity in a Jurkat cell extract (Fig. 3A), and 50–100 µg/ml TA was needed for a similar inhibitory potency in intact Jurkat T cells (Fig. 4). It suggests that higher concentrations of TA are required for inhibiting cellular proteasome activity *in vivo*. This argument is in agreement with the fact that higher concentrations of other proteasome inhibitors, even specific ones, are also needed to inhibit the proteasome in cells, e.g., the IC<sub>50</sub> value of the specific proteasome inhibitor  $\beta$ -lactone to inhibit the chymotrypsin-like activity of purified 20S proteasome was 0.1–0.6 µM (23, 29). However, when used in intact Jurkat T cells,  $\beta$ -lactone at 10 µM inhibited only 20% of the proteasomal chymotrypsin-like activity (23). Also, the IC<sub>50</sub> value of the proteasome inhibitor LLnL to inhibit a purified 20S proteasomal chymotrypsin-like activity was 0.14 µM (30), but 10 µM inhibited only 40% of chymotrypsin-like activity in living Jurkat cells (23). Furthermore, we and other researchers also reported that concentrations of dipeptidyl proteasome inhibitors to inhibit purified 20S proteasome were ~500 times lower than those to inhibit the living cell proteasome activity (15, 31). Finally, tea polyphenol (–)-epigallocatechin-3-gallate showed greater potencies to purified 20S proteasome (IC<sub>50</sub> 86 nM) than to intact cellular proteasome activity (24% inhibition at 10 µM; Ref. 23).

The concentrations (10–100 µg/ml) of TA we used in Jurkat T cells are similar to those other researchers used in various cell culture systems, e.g., TA at 50–200 µg/ml concentration was shown to be able to inhibit human immunodeficiency virus promoter activity induced by 12-O-Tetra De-

canoylphorbol-13- $\beta$ -acetate in Jurkat T cells (32). In addition, TA at a concentration between 12.5 and 50  $\mu$ g/ml suppressed 50% of cell growth of isolated human malignant tumors (8). The physiological levels of TA in human or animal bodies are currently unknown. Nepka *et al.* (6) reported that by feeding C3H male mice bearing hepatoma with TA-containing drinking water, TA at 75, 150, and 300 mg/l (or  $\mu$ g/ml) exerted chemopreventative activity. These TA concentrations that exhibited chemopreventative activity exceeded those used in our cell culture experiments. More work is needed in this area to determine the physiological serum concentrations of TA after dietary intake.

The accumulation of p27 and Bax proteins in Jurkat T cells (Fig. 5) was attributable to inhibition of the proteasome activity by TA, which is supported by the following evidence: (a) as discussed above, TA is a relatively specific, potent proteasome inhibitor *in vitro* (Figs. 2 and 3); (b) TA inhibits the chymotrypsin-like activity of the proteasome *in vivo* (Fig. 4); and (c) accumulation of both p27 and Bax proteins was observed in both a time- and concentration-dependent manner (Fig. 5 and data not shown).

The following arguments are consistent with the idea that TA-accumulated p27 and Bax proteins are functional in Jurkat tumor cells. First, when Jurkat T cells were treated with TA, both p27 expression and G<sub>1</sub> population were increased simultaneously in a time-dependent manner (Figs. 5 and 6). This result is also consistent with previous reports that overexpression of p27 could cause growth arrest in G<sub>1</sub> (25, 26). Second, several hours after Bax accumulation (at 4 h; Fig. 5C), cell death occurred (at 12 h), as judged by increased levels of pre-G<sub>1</sub> cell population and PARP cleavage (Figs. 6 and 7). TA-induced apoptotic cell death is also time- and concentration-dependent (Figs. 6 and 7). Therefore, accumulation of Bax by TA before apoptosis is consistent with the fact that Bax acts as a cell death promoter (27, 28).

In summary, our current study has demonstrated that TA can inhibit the proteasome activity *in vitro* and *in vivo* and indicated that inhibition of the proteasome activity by TA may be a novel mechanism for its previously observed anticarcinogenic activity (3, 5–7). These studies suggest the importance of plant foods in a cancer preventative diet.

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# Therapeutic Potential of Proteasome Inhibitors in Hematologic Malignancies

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## ABSTRACT

The ubiquitin/proteasome-dependent degradation pathway plays an essential role in up-regulation of cell proliferation, down-regulation of cell death (apoptosis), and development of drug resistance in human tumor cells, suggesting the use of proteasome inhibitors as potential novel anticancer drugs. This hypothesis has been supported by both *in vitro* and *in vivo* experimental results. *In vitro*, proteasome inhibitors rapidly induce apoptosis in a variety of human cancer cell lines, selectively trigger apoptotic cell death in cancer and oncogene-transformed, but not normal or untransformed cells, and are even able to activate the cell death program in human cancer cells that are resistant to various anticancer agents. *In vivo*, proteasome inhibitors suppress tumor growth *via* induction of cancer cell apoptosis and inhibit the process of angiogenesis. Studies from my laboratory have also demonstrated that synthetic (*i.e.*, peptidyl) or natural (*i.e.*, tea polyphenols) proteasome inhibitors potently and selectively inhibit the chymotrypsin-like, but not trypsin-like, proteasomal activity in human tumor cells including leukemia. Such an inhibition of the proteasome activity in intact tumor cells leads to accumulation of several specific proliferation-inhibitory or apoptosis-inducing proteins, resulting in induction of either growth arrest or apoptosis.

## INTRODUCTION

**Dysregulation of cell death in human tumors.** One important feature of human cancers is their decreased rates of cell death (apoptosis). The phenotype associated this "too-little-death" problem is drug resistance. Too-little-death in cancer cells partially results from increased levels of some death-inhibitory (oncogenic) activities. *bcr-abl* is one of the death inhibitor classes of oncogenes. Overexpression of *bcr-abl* is found in chronic myelogenous leukemia (1), resulting in resistance of these cells to apoptosis induced by serum deprivation, irradiation, and chemotherapeutic agents (2, 3). Overexpression of another cell death inhibitor, *bcl-2* pro-oncogene, has been found in follicular lymphomas, diffuse large cell lymphoma, chronic lymphocytic leukemia, and breast cancer (4). Too-little-cell death in cancer might have also been caused by decreased levels of some death-inducing activities (tumor suppressors). Consistent with this hypothesis, pro-apoptotic Bax protein is mutated in human colon cancer and some leukemia (5, 6). Reduced expression of Bax alone, or low ratios of Bax to Bcl-2, is associated with poor response rates to radio- or chemotherapy in patients with B-cell chronic lymphocytic leukemia (7), breast (8), ovarian (9), cervical (10) and pediatric cancers (11). Mutations in p53, a transactivator of *bax* (12), were also associated with resistance of human tumors to radio and chemotherapy (13). Finally, mutations of the cell death gene CD95 (Fas/Apo-1) were found in adult T-cell leukemia (ATL), childhood T-lineage acute lymphoblastic leukemia (T-ALL), urinary bladder carcinomas and non-small cell lung cancer (14-17), suggesting a role of *fas* mutation in drug resistance development of human cancers.

**Regulation of cell death by the proteasome.** The ubiquitin/proteasome system includes two distinct steps: ubiquitination and degradation (18, 19). Ubiquitination is the step after which the target protein is tagged by multiple ubiquitin molecules and, therefore, can be selectively recognized by the proteasome complex from other proteins. Degradation of such multi-ubiquitinated proteins occurs on a large 26S proteasome complex (18, 19). Recent studies have demonstrated that the ubiquitin/proteasome-mediated protein degradation pathway plays an important role in regulating cell death process. This suggests that degradation of some specific cellular proteins plays an essential role in determining whether a cell proliferates or dies. In this article, I will summarize our studies on the proliferation-inhibitory, cancer-preventative and anti-tumor activities of some synthetic and natural proteasome inhibitors. I will also discuss the involved molecular mechanisms, potential proteasome target proteins, and the clinical relevance.

## INDUCTION OF APOPTOSIS IN HUMAN CANCER CELLS BY PROTEASOME INHIBITOR TREATMENT

We have reported that a synthetic, novel dipeptidyl proteasome inhibitor, CEP1612 [phthalimide-(CH<sub>2</sub>)<sub>8</sub>CH(cyclopentyl)CO-Arg(NO<sub>2</sub>)-Leu-H], at low concentrations rapidly activates caspases and induces apoptosis in HL-60 and Jurkat T cells (20). Since these two cell lines do not contain functional p53 gene, the induced apoptosis must be p53-independent. The apoptosis-inducing potencies of dipeptidyl proteasome inhibitors

match precisely their order of inhibition of the proteasome chymotrypsin-like activity. CEP1612 has greater apoptosis-inducing potency than etoposide and is able to induce apoptosis in human prostate, breast, tongue and brain tumor cell lines. The proteasome inhibitor-induced apoptosis is associated with accumulation of cdk inhibitors p21 and p27 (20).

Recent experiments have demonstrated that apoptosis can be triggered by release of mitochondrial cytochrome c into cytosol, which in turns induces activation of effector caspase pathway (21). To study whether proteasome inhibitor-induced apoptosis is cytochrome c-dependent, we treated human Jurkat T cells with the tripeptidyl proteasome inhibitor LLnV, followed by measurement of apoptotic death, PARP cleavage and cytochrome c release. The LLnV-induced PARP cleavage started at 4 h. Levels of cytosolic cytochrome c were also increased at 4 h after the addition of LLnV, and further increased afterwards. The kinetics of cytochrome c release is similar to that of PARP cleavage, suggesting that proteasome inhibition-induced apoptosis is cytochrome c-dependent.

We hypothesized that Bax is a direct target protein of the ubiquitin/proteasome pathway and that inhibition of this pathway by a proteasome inhibitor should accumulate Bax protein that would in turn induce cytochrome c release. To test this hypothesis, we measured the levels of Bax protein by Western blotting. Expression of Bax protein was increased after 1 h LLnV treatment, and further increased afterwards. Therefore, increased levels of Bax protein after LLnV treatment occur prior to release of cytochrome c and induction of apoptosis.

We also measured the subcellular localization of Bax by immunohistochemistry. In untreated Jurkat cells Bax protein was primarily located in the cytoplasm. Treatment with lactacystin markedly increased Bax-associated immunofluorescence in the cytoplasm, which was consistent with the results obtained from Western blotting. The increased Bax-associated immunofluorescence remained largely clustered around the nuclei, suggesting accumulation of Bax in mitochondria. When cells were treated with LLM, a weak proteasome inhibitor but a strong calpain inhibitor, none of the above events, including cell death, PARP cleavage, cytochrome c release and Bax accumulation, was observed (22).

If Bax is a direct target of the ubiquitin-proteasome pathway, inhibition of proteasome activity should result in accumulation of ubiquitinated Bax protein. To investigate this possibility, protein extracts of Jurkat T cells treated with lactacystin or LLnV were immunoprecipitated with a monoclonal anti-Bax antibody. The prepared Bax immunoprecipitates were then analyzed by a Western blot assay using a polyclonal anti-ubiquitin antibody. Several polypeptide bands including p55 and p47 were detected in the untreated cell lysate. Treatment with lactacystin or LLnV increased levels of both p55 and p47 levels, suggesting that they are polyubiquitinated forms of Bax. To investigate whether proteasome inhibition might also affect Bax mRNA levels, we measured Bax mRNA in lactacystin or LLnV-treated human Jurkat T cells by RT-PCR. No up-regulation on Bax mRNA was detected during the process of proteasome inhibition (22). Taken together, our data indicate that Bax protein degradation is mainly regulated by the ubiquitin/proteasome pathway and that inhibition of this pathway leads to Bax accumulation, resulting in cytochrome c-dependent apoptosis. Previously, another group also reported that Bax protein levels were increased when HeLa or Saos-2 cells were

treated with a proteasome inhibitor although its relationship to apoptosis was not investigated (23).

We have also shown that ester bond-containing tea polyphenols [such as (-)-epigallocatechin-3-gallate or EGCG] potently and specifically inhibit the chymotrypsin-like activity of the proteasome *in vitro* (IC<sub>50</sub> 86-194 nM) and *in vivo* (1-10  $\mu$ M) at the concentrations found in the serum of green tea drinkers. Atomic orbital energy analyses and high-performance liquid chromatography suggest that the carbon of the polyphenol ester bond is essential for targeting and thereby inhibiting the proteasome in cancer cells. This inhibition of the proteasome by EGCG in several tumor and transformed cell lines results in accumulation of two natural proteasome substrates, p27<sup>Kip1</sup> and I $\kappa$ B- $\alpha$ , followed by growth arrest in the G<sub>1</sub> phase of the cell cycle (24). EGCG at higher concentrations induced apoptosis in leukemia and prostate cancer cell lines, associated with accumulation of Bax protein (unpublished result). Our study suggests that the proteasome is a cancer-related molecular target of tea polyphenols and that inhibition of the proteasome activity by ester bond-containing polyphenols may contribute to the previously observed cancer preventative effect of tea (25-27).

### DRUG-RESISTANT TUMOR CELLS ARE SENSITIVE TO PROTEASOME INHIBITORS

Although systemic chemotherapy and irradiation are currently the preferred ways of treating human tumors, acquired or de novo drug resistance prevents a satisfactory outcome of such treatments. Understanding the molecular mechanisms of drug resistance in human cancers is essential for improving current therapies.

**Bcl-2-overexpressing tumor cells.** Many human cancer cells overexpressing *bcl-2* oncogene are resistant to radiotherapy and chemotherapy (28). We found that proteasome inhibitors induced apoptosis in human leukemia Jurkat T cells overexpressing Bcl-2 (20). Similarly, another group also reported that proteasome inhibitors activated apoptotic death program in Bcl-2 overexpressing prostate cancer cells, which are also resistant to cytotoxic anti-cancer drugs (29). We found that after exposure to 30  $\mu$ M CEP1612 for 3.5 h, ~100% of the Bcl-2-overexpressing Jurkat cells, similar to the vector-transfected cells, exhibited the apoptosis-specific nuclear morphology. In contrast, etoposide failed to induce apoptosis in Bcl-2-expressing cells. Failure of Bcl-2 to inhibit CEP1612-induced apoptosis could be due to the great potency of CEP1612 at the applied concentration. To investigate this possibility, the control vector cells were exposed to 15  $\mu$ M CEP1612, a treatment that was less potent than 50  $\mu$ M etoposide. Overexpression of Bcl-2 had much less inhibitory effects on the cells treated with CEP1612 than those with etoposide. Although a slightly delayed PARP cleavage was observed in Bcl-2-expressing cells treated with CEP1612 for 5 to 8 h, similar levels of PARP cleavage were detected in both Bcl-2-expressing and the vector cells after 10 h-treatment. By comparison, expression of Bcl-2 almost completely inhibited PARP cleavage induced by a treatment of 50  $\mu$ M etoposide for up to 11 h (20). It appears, therefore, that the dipeptidyl proteasome inhibitor CEP1612 initiates the apoptotic death program through a pathway that bypasses Bcl-2 inhibitory function.

It is possible that proteasome inhibition results in accumulation of Bax in mitochondria, which binds to and inhibits Bcl-2 protein, triggering cytochrome c-dependent apoptosis (21). To investigate this possibility, we measured levels of cytochrome c and Bax in Bcl-2-overexpressing cells after proteasome inhibitor treatment. Consistent with the partial inhibition of PARP cleavage by Bcl-2 observed earlier, release of mitochondrial cytochrome c in Bcl-2-transfected cells was also inhibited slightly in 4 hours after treatment. However, very high levels of cytochrome c were found at later time points in both cell lines. Bax protein was accumulated in both Bcl-2-expressing and vector cells. Proteasome inhibition in Bcl-2-expressing cells caused accumulation of Bax protein primarily in mitochondria and increased levels of Bax-associated Bcl-2 protein, supporting the argument that Bax is responsible for inhibiting mitochondrial Bcl-2 and releasing cytochrome c. Bcl-2 protein did not undergo any post-translational modifications, phosphorylation or cleavage, during proteasome inhibition (22). Therefore, accumulation of Bax protein by proteasome inhibition is likely responsible for the ability of a proteasome inhibitor to overcome Bcl-2-mediated protection from apoptosis.

**Bcr-Abl-overexpressing leukemia cells.** The chimeric oncoprotein Bcr-abl, a protein tyrosine kinase, mediates cellular transformation and multiple drug resistance (30). We have found that proteasome inhibition is sufficient to induce cytochrome c-dependent apoptosis in K562 human chronic myelogenous leukemia cells that overexpress Bcr-Abl oncoprotein (31 and unpublished results). Our data have identified two major mechanisms involved: inhibition of Bcr-Abl function (31) and accumulation of Bax protein (22).

The level of Bcr-Abl/p210 protein expression was high in exponentially growing K562 cells, as detected by immunoblotting with an anti-c-Abl antibody (derived from a fusion protein corresponding to the carboxyl region of the *v-abl* protein). However, the Bcr-Abl protein expression was slightly decreased after treatment with 50  $\mu$ M LLnV for 4 h. A significant reduction in Bcr-Abl protein expression was observed after 8 h treatment. In contrast, no or little change in expression of c-Abl/p145 protein was observed in the same samples. When similar lysates were immunoblotted with an anti-Bcr antibody that recognized the amino terminal sequence of Bcr, significant reduction in Bcr-Abl expression was again observed, while the level of Bcr/p160 was only slightly reduced. Importantly, after 8 h-treatment, no apoptotic changes such as PARP cleavage were observed. The level of Bcr-Abl protein expression remained low after 12 h treatment, and further decreased after 24 h. The process of PARP cleavage started at 12 h and was completed at 24 h. The ability of LLnV to reduce the Bcr-Abl protein expression is about 100-fold greater than that of LLM, which corresponded exactly to their potencies in induction of PARP cleavage. Furthermore, when the specific proteasome inhibitor lactacystin was used, the decrease in the Bcr-Abl levels was again observed prior to apoptosis induction. In contrast, treatment with the specific cysteine protease inhibitor E-64d neither reduced Bcr-Abl protein level nor induced apoptosis (31). These results demonstrate that inhibition of the proteasome activity leads to a significant reduction of Bcr-Abl protein expression and subsequent induction of apoptosis.

To establish that the decreased Bcr-Abl expression leads to attenuation of Bcr-Abl-mediated protein tyrosine phosphorylation, the lysates, which were prepared from K562 cells treated with 50  $\mu$ M LLnV for 7 h, were also analyzed by immunoblotting with an



anti-phosphotyrosine antibody. Several heavily tyrosine-phosphorylated proteins were observed in control K562 cell lysates, which is typical of Bcr-Abl-expressing cells (32). A band of ~210 kDa is Bcr-Abl itself since Bcr-Abl undergoes autophosphorylation on tyrosine residues (33) and since this band was not detected in HL-60 cell lysates. After 7 h LLnV treatment, the levels of tyrosine phosphorylation for nearly all of the major phosphotyrosine-containing proteins, as well as Bcr-Abl itself, were significantly reduced (31). This demonstrates that LLnV-mediated reduction of Bcr-Abl protein expression in K562 cells leads to inactivation of Bcr-Abl tyrosine kinase function, which is responsible for the decrease in level of Bcr-Abl-mediated protein tyrosine phosphorylation prior to the initiation of apoptotic execution. The molecular mechanisms responsible for proteasome inhibition-induced decrease in Bcr-Abl oncoprotein levels remain unknown. We have found that it is not inhibited by a variety of protease inhibitors, which suggests that the Bcr-Abl level reduction, if due to proteolytic degradation, must be mediated by some unique protease(s). Alternatively, a different mechanism may be responsible. One possibility is that proteasome inhibition might lead to inhibition of *bcr-abl* transcription.

We investigated whether Bax accumulation by proteasome inhibition also contributes to overcoming Bcr-Abl-mediated protection against apoptosis. We found that cytochrome c was released after 12 h treatment with LLnV, prior to cleavage of PARP at 16 h. The levels of Bax/p21 protein was increased at as early as 4 h and reached the peak level at 12 h post-treatment. A polypeptide(s) of 47 kDa was accumulated prior to cytochrome c release, which might be an ubiquitinated form of Bax since a similar polypeptide was detected by antibodies to Bax and ubiquitin in our immunoprecipitation experiment (unpublished data). These results were also in agreement with the immunostaining data, which showed a dramatic increase of Bax immunofluorescence signal in mitochondria following proteasome inhibition. Therefore, both inactivation of Bcr-Abl function and accumulation of Bax protein are responsible for the ability of a proteasome inhibitor to overcome Bcr-Abl-mediation protection from apoptosis. Other groups have also reported that lactacystin treatment sensitized chemo- and radio-resistant CLL cells to TNF- $\alpha$ -induced apoptosis (34, 35).

## SUPPRESSION OF HUMAN TUMOR GROWTH BY PROTEASOME INHIBITORS IN XENOGRAFT MODELS

Given that the proteasome is required for proliferation and survival of tumor cells, inhibition of its activity should arrest or retard cancer progression *in vivo* via inhibition of cell cycle progression and/or induction of apoptosis. Indeed, several groups including ours recently have extended the studies of anticancer activity of proteasome inhibitors from *in vitro* human cancer cell lines to *in vivo* human xenograft animal models (36-38).

We reported (38) that the dipeptidyl proteasome inhibitor, CEP1612, induced apoptosis and inhibited tumor growth of the human lung cancer cell line A-549 in an *in vivo* model. In cultured A-549 cells, CEP1612 treatment resulted in accumulation of two proteasome natural substrates, the cyclin dependent kinase inhibitors p21<sup>WAF1</sup> and p27<sup>KIP1</sup>, indicating its ability to inhibit proteasome activity in intact cells. Furthermore, CEP1612 induced apoptosis as evident by caspase-3 activation and poly(ADP-ribose) polymerase cleavage. Treatment of A-549 tumor bearing (s.c.) nude mice with CEP1612

(10 mg/kg per day, *i.p.* for 31 days) resulted in massive induction of apoptosis (TUNEL) and significant 68% tumor growth inhibition ( $p < 0.05$ ). Furthermore, immunostaining of tumor specimens also demonstrated *in vivo* accumulation of p21<sup>WAF1</sup> and p27<sup>KIP1</sup> following CEP1612 treatment. The results suggest that CEP1612 is a promising candidate for further development as an anticancer drug and demonstrate the feasibility of using proteasome inhibitors as novel antitumor agents.

## CONCLUSIONS

The involvement of the ubiquitin/proteasome-dependent degradation pathway in up-regulation of cell proliferation, down-regulation of cell death, and development of drug resistance in tumor cells suggests the potential use of proteasome inhibitors as novel anticancer drugs. This hypothesis has been supported by both *in vitro* and *in vivo* experimental results as well as Phase I clinical results (39, 40).

Given the fact that the proteasome is involved in several important cellular processes, it will be a great challenge for researchers to design specific, selective and potent proteasome inhibitors. Ideally a proteasome inhibitor to be used for therapeutic purposes should not inhibit other protease activities, interact with other proteins, and affect normal cells at therapeutic doses. It would be important that the proteasome inhibitor selectively accumulates only the pro-apoptotic or anti-proliferative proteins (*i.e.*, Bax, p53, p27), but not the anti-apoptotic or pro-proliferative proteins (*i.e.*, Bcl-2, MDM2, cyclin E). Such a selectivity of the proteasome inhibitor should be associated with increased potency against tumor cells. Equally important, better understanding of the cancer-related proteasome target proteins will accelerate the drug discovery process of novel proteasome inhibitors. It is to be hoped that more specific, selective and potent proteasome inhibitors will be developed in laboratories and used in cancer therapies in the near future.

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## Review

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# Pharmacological proteasome inhibitors and their therapeutic potential

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Implication of the proteasome in human cancer, inflammation, autoimmune and other diseases has prompted design, synthesis and evaluation of various pharmacological proteasome inhibitors. In this review, we will summarise the literature related to human cancer cell death induction by proteasome inhibitors, examine the proteasome inhibitor patents published over the past five years and evaluate the potential clinical applications in the use of proteasome inhibitors for treating cancer and other human diseases.

**Keywords:** *Alzheimer's disease, autoimmune disease, bone disease, cancer, inflammation, muscle wasting disease, neurological disease, Parkinson's disease*

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## 1. Introduction

The ubiquitin/proteasome degradation pathway plays an essential role in major cellular processes, including antigen presentation, cell proliferation, differentiation and apoptosis [1-4]. Attachment and conjugation of ubiquitin to the target protein is the first step that is required in the ubiquitin/proteasome pathway. Such multi-ubiquitinated proteins are then degraded by a large 26S proteasome complex, which is composed of a 20S catalytic core and associated regulatory proteins [1-4]. The proteasome has been implicated in cancer and other human diseases [5-9], suggesting that proteasome inhibitors have therapeutic potential. This idea has attracted researchers to design, synthesise and biologically evaluate a variety of pharmacological inhibitors of the proteasome.

## 2. Literature related to induced cancer cell death by proteasome inhibitor

It has been well documented that the ubiquitin/proteasome pathway plays a key role in the degradation of regulatory proteins involved in cell cycle progression and cell proliferation [1-4]. In addition, recent studies using proteasome inhibitors have suggested that the proteasome also plays a role in the downregulation of human cancer cell death (see the following references). Experiments using cell cultures and mouse models have demonstrated that proteasome inhibitors are able to (i) induce apoptosis selectively in human tumour but not normal cells, (ii) overcome tumour cell resistance to cytotoxic therapies and (iii) inhibit tumour growth in xenograft models.

### 2.1 Proteasome inhibitors selectively target tumour and the transformed cells, but not normal cells

As one of the first reports, Imajoh-Ohmi *et al.* observed induction of apoptosis in human leukemia U937 cells after a treatment with 5  $\mu$ M lactacystin [10], a microbial metabolite and a specific proteasome inhibitor [11,12]. Fujita *et al.* reported that TNF-induced caspase-3 activity was increased by 5  $\mu$ M lactacystin or a tripeptide proteasome inhibitor, benzyloxycarbonyl-Leu-Leu-leucinal (LLL or MG132) or *N*-carbobenzoxy-Leu-Leu-norvalinal (LLnV or MG115), but not by the cysteine protease inhibitor E-64d. The proteasome inhibitor alone, however, was not able to activate the caspase-3 activity in U937 cells [13]. Shinohara *et al.* also demonstrated that the tripeptide proteasome inhibitors LLL and *N*-carbobenzoxy-Leu-Leu-norleucinal (LLnL or MG101), but not the calpain inhibitor benzyloxycarbonyl-Leu-leucinal, induced programmed cell death and caused the accumulation of p53 protein in human Molt-4 leukaemia cells [14]. Lopes *et al.* reported apoptotic death of Rat-1 fibroblasts and PC12 pheochromocytoma cells after treatment with the proteasome inhibitor LLnV or Z-Ile-Glu(O-t-butyl)-Ala-leucinal (PSI), but not with inhibitors of lysosomal proteases or an alcohol analogue of PSI, Z-Ile-Glu(O-t-butyl)-Ala-leucinol. This proteasome inhibitor-induced apoptosis in Rat-1 and PC12 cells was p53-dependent [15].

We also reported that a novel dipeptidyl proteasome inhibitor, CEP1612 [phthalimide-(CH<sub>2</sub>)<sub>8</sub>CH(cyclopentyl)CO-Arg(NO<sub>2</sub>)-Leu-H], at low concentrations rapidly activated caspases and induced apoptosis in HL-60 and Jurkat T-cells in a p53-independent manner. The apoptosis-inducing potencies of CEP1612 and its analogues matched precisely their order of inhibition of the proteasome chymotrypsin-like activity. CEP1612 had greater apoptosis-inducing potency than etoposide and was able to induce apoptosis in human prostate, breast, tongue and brain tumour cell lines. The proteasome inhibitor-induced apoptosis was associated with accumulation of the cdk inhibitors p21 and p27 [16].

Consistent with the above findings that proteasome inhibitors have apoptosis-inducing activity in tumour cells, the antitumour compound eponemycin [17] was found to selectively target the 20S proteasome [18]. Dihydroeponemycin covalently modifies proteasome subunits LMP2 and LMP7 and inhibits three major

peptidolytic activities of the proteasome at different rates, resulting in a spindle-like cellular morphological change and apoptosis [18].

We also found that the proteasome inhibitor CEP1612 induced apoptosis selectively in simian virus 40 (SV40)-transformed, but not in the parental WI-38, normal human fibroblasts [16]. CEP1612 treatment increased p21 levels ~10-fold in both the transformed and normal WI-38 cells. However, the same CEP1612 treatment resulted in an 8-fold increase in p27 levels only in the transformed, but not in normal, WI-38 cells. In addition, LLnV treatment also increased the level of p27 in the transformed cells 10-fold higher than that in the normal cells [16]. This finding suggests that proteasome inhibition increases p27 expression, which is accompanied by selective initiation of an apoptotic death program in the transformed but not in normal human fibroblasts. Similarly, Orlowski *et al.* also reported that fibroblasts transformed with *ras* and *myc*, lymphoblasts transformed by *c-myc* alone and a Burkitt's lymphoma cell line that overexpress *c-myc* were up to 40-fold more susceptible to apoptosis induced by Z-LLF-CHO (a cell-permeable peptidyl aldehyde inhibitor of the chymotrypsin-like activity of the proteasome) than were primary rodent fibroblasts or immortalised non-transformed human lymphoblasts [19]. In addition, Delic *et al.* [20] and Masdehors *et al.* [21] reported that the specific proteasome inhibitor lactacystin was able to activate the apoptotic pathway in chronic lymphocytic leukaemia (CLL), but not in normal cells. Lactacystin treatment also sensitised chemo- and radio-resistant CLL cells to TNF- $\alpha$ -induced apoptosis [20,21]. Taken together, these data suggest that proteasome inhibitors are able to selectively induce apoptotic death in tumour and oncogene-transformed cells.

### 2.2 Proteasome inhibitors are able to activate the apoptotic death program in drug-resistant tumour cells

It has been suggested that proteasome-mediated degradation of some apoptosis activators play an important role in the development of drug resistance of human cancers [3,22]. If true, then proteasome inhibitors should be able to overcome the drug resistance of these human cancer cells. Indeed, we found that proteasome inhibitors were able to induce apoptosis in human leukaemia Jurkat T-cells overexpressing Bcl-2 that were resistant to chemotherapy and radiotherapy [16]. Similarly, Herrmann *et al.* reported that proteasome inhibitors activated the

apoptotic death program in Bcl-2 overexpressing prostate cancer cells [23]. The ability of a proteasome inhibitor to overcome Bcl-2-mediated protection from apoptosis suggests that a Bcl-2 inhibitory protein could be a target of the proteasome. Indeed, most recently, we reported that Bax, a Bcl-2 binding inhibitor and a cell death inducer, was a target of the ubiquitin/proteasome-mediated degradation pathway. The proteasome inhibitor treatment accumulated Bax protein in the mitochondria, leading to cytochrome c release, caspase activation and apoptosis [24].

We have also reported that proteasome inhibitors were able to overcome Bcr-Abl-mediated protection from apoptosis [25], which involves two major mechanisms: accumulation of Bax protein and inhibition of Bcr-Abl function. In addition, Fanelli *et al.* [26] and Chandra *et al.* [27] found that proteasome inhibition resulted in an increased tumour cell sensitivity to retinoic acids or glucocorticoids, respectively. Furthermore, Ogiso *et al.* [28] showed that proteasome inhibitors were able to increase the toxicity of topoisomerase II-directed drugs (e.g., etoposide and doxorubicin) in resistant solid tumour cells, whereas proteasome inhibition had no effect against the non-topoisomerase II-targeted drugs (e.g., methotrexate and vincristine). The ability of proteasome inhibitors to sensitise these drug-resistant tumour cells correlated well with their ability to inhibit depletion of topoisomerase II  $\alpha$  induced by glucose deprivation [28]. All these findings are consistent with the hypothesis that proteasome-mediated degradation of some apoptosis activators plays an important role in the development of drug resistance of human cancers.

### 2.3 Proteasome inhibitors are able to inhibit human tumour growth in xenograft models

Given that the proteasome is required for proliferation and survival of tumour cells [1-5], inhibition of its activity should arrest or retard cancer progression *in vivo* via inhibition of cell cycle progression and/or induction of apoptosis. Indeed, Orlowski *et al.* [19] found that after injecting interscapularly into severe combined immunodeficiency mice bearing sc. Burkitt's lymphoma tumours, single doses of the proteasome inhibitor Z-LLF-CHO induced statistically significant early tumour regression and a significant delay in tumour progression. Analysis of tumour specimens revealed enhanced apoptotic cell death in Burkitt's lymphoma tumours from mice treated with

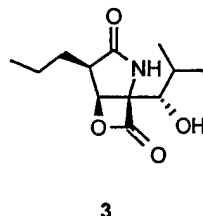
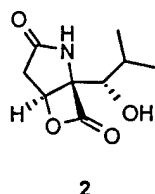
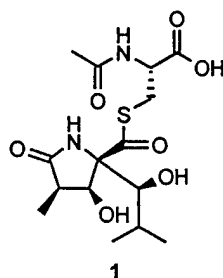
Z-LLF-CHO. By contrast, when the peptidyl alcohol Z-LLF-OH that does not inhibit proteasome activity was used *in vivo*, neither inhibition of tumour progression nor induction of apoptosis was detected. Their results, showing a 42% tumour growth delay [19], indicate that proteasome inhibitors have the potential for suppressing *c-myc*-mediated tumour growth.

Adams *et al.* [29] also reported that following weekly iv. treatment of PS-341, a dipeptidyl boronate proteasome inhibitor, to mice with PC-3 tumours, a 60% decrease in tumour volume was detected *in vivo*. Direct injection of the proteasome inhibitor into the tumour also caused a 70% decrease in tumour burden, resulting in 40% of the drug-treated mice having no detectable tumours at the end of the study. Iv. administration of radioactive PS-341 resulted in a rapid and widespread distribution of PS-341, with the highest levels accumulating in the liver and GI tract, lowest levels in the skin and muscle, modest levels in the prostate and no apparent penetration in the CNS. By assaying the biological proteasome inhibitory activity of PS-341, it was found that PS-341 was able to penetrate PC-3 tumours and inhibit intracellular proteasome activity 1.0 h after iv. delivery of the drug [29]. Therefore, a proteasome inhibitor such as PS-341 reaches and inhibits its biological target, the proteasome, under *in vivo* conditions.

Currently, Phase I studies of PS-341 are being conducted in patients with advanced malignancies [30,31]. No toxicity has been observed so far. As predicted, at 1 h after drug infusion, proteasome inhibition was achieved. The percent of proteasome inhibition was consistent from patient to patient. Responses were seen at low levels of PS-341 suggesting therapeutic index [30,31]. The evidence that PS-341 has clinical activity without toxicity has provided strong support for proof-of-concept of using proteasome inhibitors as potential therapeutic anticancer drugs.

Recently, it has been suggested that the ubiquitin/proteasome pathway is also involved in the process of angiogenesis [32,33]. By using an *in vivo* angiogenesis model, lactacystin was found to have an anti-angiogenic activity although the exact molecular mechanism remains unknown [32]. In contrast, PR39, a macrophage-derived peptide, acts as a potent angiogenesis inducer *via* inhibiting the ubiquitin/proteasome-dependent degradation of hypoxia-inducible factor-1 $\alpha$  protein [33]. Under some





*in vivo* conditions proteasome inhibitors have anti-angiogenic activity [32] and inhibition of tumour angiogenesis is an important strategy to suppress tumour growth [34,35], therefore, it is possible that inhibition of angiogenesis by proteasome inhibitors contributes to their *in vivo* antitumour activity.

### 3. Patents on proteasome inhibitors: 1996 - 2000

In the past five years, many structurally unrelated proteasome inhibitors, which we divided into several classes below, have been patented and their potential application in different human diseases has been claimed.

#### 3.1 Lactacystin and its analogues

Lactacystin, a non-peptide proteasome inhibitor [12], was identified as a *Streptomyces* metabolite [11]. Lactacystin, when converted to its active form *clasto* lactacystin  $\beta$ -lactone, is a highly specific and irreversible inhibitor of the proteasome [36-38]. This  $\beta$ -lactone contains an ester bond that is responsible for interacting with the nucleophilic hydroxyl group of the threonine residue in the proteasome, resulting

in the inhibition of proteasomal activity [39]. Lactacystin is a much more potent inhibitor of the chymotrypsin-like activity ( $IC_{50} = 0.8 \mu M$ ) than the trypsin-like ( $IC_{50} = 10 \mu M$ ) or peptidyl-glutamyl peptide hydrolysing (PGPH) ( $IC_{50} > 100 \mu M$ ) activities of the proteasome [38,40]. Lactacystin does not inhibit the activities of serine or cysteine proteases, but has been found recently to have some inhibitory activity to cathepsin A activity [41].

Several patents have been filed in which synthetic methods and potential clinical usage of lactacystin and its analogues were claimed. Harvard College has claimed 18 novel analogues of lactacystin (**1**) and  $\beta$ -lactone (**2**) for their use in the treatment of inflammation, cancer, psoriasis, restenosis, Alzheimer's, cachexia and muscle wasting diseases [101]. Several of these compounds were shown to induce neurite outgrowth in Neuro 2A neuroblastoma cells, inhibit cell cycle progression in MG-63 osteosarcoma cells and prevent protein degradation in mouse myoblast C2C12 cells [101]. ProScript has claimed the use of a  $\beta$ -lactone analogue (compound **3**) and another related compound in the treatment of stroke and myocardial infarction by determining the ability of two preferred NF- $\kappa$ B inhibitors (which are also proteasome inhibitors) to protect against ischaemia in a rat model [102]. The specified compound reduced infarct volume by 70% and neurological impairment by 40% when administered as a bolus dose of 0.1 mg/kg iv. at 2 h after the start of occlusion. ProScript also specifically claimed the syntheses of several lactacystin analogues including compound **3** and their use either alone or in conjunction with glucocorticoids for the treatment of multiple sclerosis and asthma [103]. The inventors found that in a rat model of experimental allergic encephalomyelitis, the specified compound at 0.3 and 1 mg/kg reduced both the relapse and the disease severity. In an allergic Brown Normal rat model, ovalbumin-induced accumulation of leukocytes in the lung was inhibited by dexamethasone or a combination of budesonide and the lactacystin analogue (but not each alone).

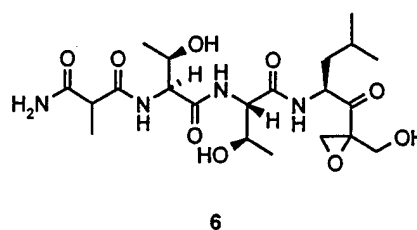
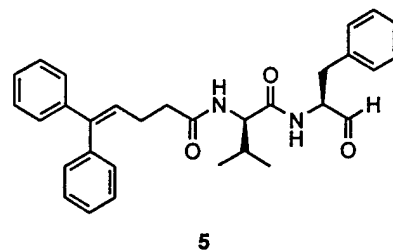
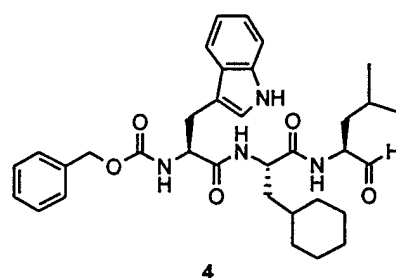
In addition, Centre de Recherche du Centre Hospitalier de l'Universite de Montreal claimed that lactacystin (**1**) treatment in conjunction with a specified composition reversed an adverse immune response *via* disruption of mitochondrial function by blocking electron transport and/or inducing mitochondrial cytochrome c release [104]. The reversed immune response was found after graft transplantation or in autoimmune disease, cancer or

inflammation. The inventors also found that lactacystin strongly inhibited T- and B-cell proliferation and induced apoptosis of activated normal and leukaemic T-cells as well as Jurkat cells. Soucy *et al.* claimed a method for synthesis of lactacystin (1) and 7-propyl-*clasto*-lactacystin- $\beta$ -lactone (3), which had fewer steps and greater yield than the previously described routes [105]. The inventors also claimed the use of these proteasome inhibitors in the treatment of diseases mediated directly (i.e., muscle wasting disease) or indirectly by the proteasome. In a rat middle cerebral artery occlusion model, the specified compound (0.3 mg/kg iv.) reduced infarct volume by 50% and improved neurological deficit by 60% compared to control. Osteoscreen disclosed 15 compounds including lactacystin (1) and claimed that a proteasome inhibitor such as lactacystin could be used for enhancing bone formation, treating a pathological dental condition, or treating a degenerative joint disorder. The specified compound exhibited ED<sub>50</sub> values of 1, 1 and 1.5  $\mu$ M against luciferase, bone formation and proteasome activities, respectively [106].

### 3.2 Peptidyl aldehydes

Peptidyl aldehyde analogues are reversible proteasome inhibitors. The aldehyde moiety of these proteasome inhibitors forms a hemi-acetyl adduct with the nucleophilic hydroxyl group of the N-terminal threonine in the proteasome [39,42]. The aldehyde group has also been shown to react with thiol groups of cysteine proteases such as cathepsin B and calpains [40,41].

ProScript claimed synthesis of formylated peptidyl derivatives (total 138 compounds disclosed, including compound 4) and their use in the treatment of muscle weakness and cachexia [107]. These compounds were claimed to inhibit MHC-I antigen presentation, p53 degradation, intracellular protein breakdown and muscle loss. The  $K_i$  value of compound 4 against 20S proteasome activity was 80 pM. Takeda Yakuhin Kogyo KK disclosed 13 novel dipeptidyl compounds and claimed the method of their production and use for the prevention and treatment of inflammation, autoimmune disorders, neurological disease and cancer [108]. The most potent compound (5) of those claimed had an IC<sub>50</sub> value of 0.17  $\mu$ M and showed a significant inhibition on adjuvant arthritis disease at a dose of 10 mg/kg p.o.



### 3.3 Epoxypeptides

Another class of proteasome inhibitors, the epoxypeptides, was originally isolated from a strain of *Streptomyces hygroscopicus* [17,43]. Epoxypeptides contain  $\alpha'$ ,  $\beta'$ -epoxyketone groups. The nucleophilic hydroxyl group on the proteasome's threonine residue attacks the  $\alpha'$ -keto group of an epoxypeptide, forming a morpholino product (an irreversible and covalent adduct), leading to inhibition of the proteasome [44]. Dihydroeponemycin, an analogue of epoxypeptides, does not inhibit calpains and trypsin, but does have some inhibitory activity towards cathepsin B [18].

Sumitomo Seiyaku KK disclosed two novel epoxypeptides, including compound 6, as proteasome inhibitors and claimed their use for the treatment of autoimmune disease, inflammatory bowel disease, asthma, Alzheimer's disease and chronic rheumatoid arthritis [109]. The IC<sub>50</sub> value of compound 6 against the proteasome is 0.22  $\mu$ g/ml, in an *in vitro* assay.

### 3.4 Peptidyl boronates

Boronate peptides are much more potent inhibitors of the proteasomal activity than the structurally corresponding aldehyde peptides [29]. The boronate moiety reversibly binds to the nucleophilic hydroxyl group of the proteasome's threonine residue, generating a stable tetrahedral intermediate [45]. Boronate peptides are also known as serine protease inhibitors [46]. Boronate peptides do not inhibit cysteine proteases probably due to the formation of a weak bond between the thiol groups and borons [45].

Cephalon claimed a novel method for inducing the death of transformed and multiple cancer cell lines, including breast, prostate, tongue, brain, lung, pancreatic, ovarian and skin, by using a boronate dipeptide proteasome inhibitor. Ten analogues were specifically claimed [110]. *In vivo* studies showed that compound **7** inhibited the growth of Lewis lung and AT-2 rat prostatic carcinoma tumour in female athymic nude mice, when administrated at 2 mg/kg sc. for 5 days per week, for 12 or 15 days.

### 3.5 Peptidyl indanones

While most of the proteasome inhibitors act by covalently modifying the N-terminal active threonine residue of the proteasome, indanone inhibitors inhibit the proteasomal activity by binding to the proteasome substrate binding sites [40,47]. The indanone moiety of these proteasome inhibitors is an electrophilic head group, which is coupled to a di or tri-peptidyl analogue that specifically binds to substrate binding sites [40,47]. Indanone derivatives selectively inhibit the chymotrypsin-like activity over other proteasomal and calpain activities [48].

CV Therapeutics claimed indanone derivatives as proteasome inhibitors and cell proliferation inhibitors [111]. The inventors described a generalised solid-phase peptide synthesis method. IC<sub>50</sub> values of 200 compounds were determined using the 20S proteasome in an *in vitro* assay. Compound **8** showed an IC<sub>50</sub> value of 0.5 µg/ml. In RAW cells, the specified compound inhibited TNF synthesis, IκB degradation, p105 to p50 processing and translocation of NF-κB to the nuclear fraction of the cell.

### 3.6 Peptidyl α-ketoamides

Lynas *et al.* [49] reported that synthetic di- and tri-peptidyl α-keto aldehydes (glyoxals) were potent inhibitors of the chymotrypsin-like activity of the proteasome, with final *K<sub>i</sub>* values of ~ 3.0 nM. CV

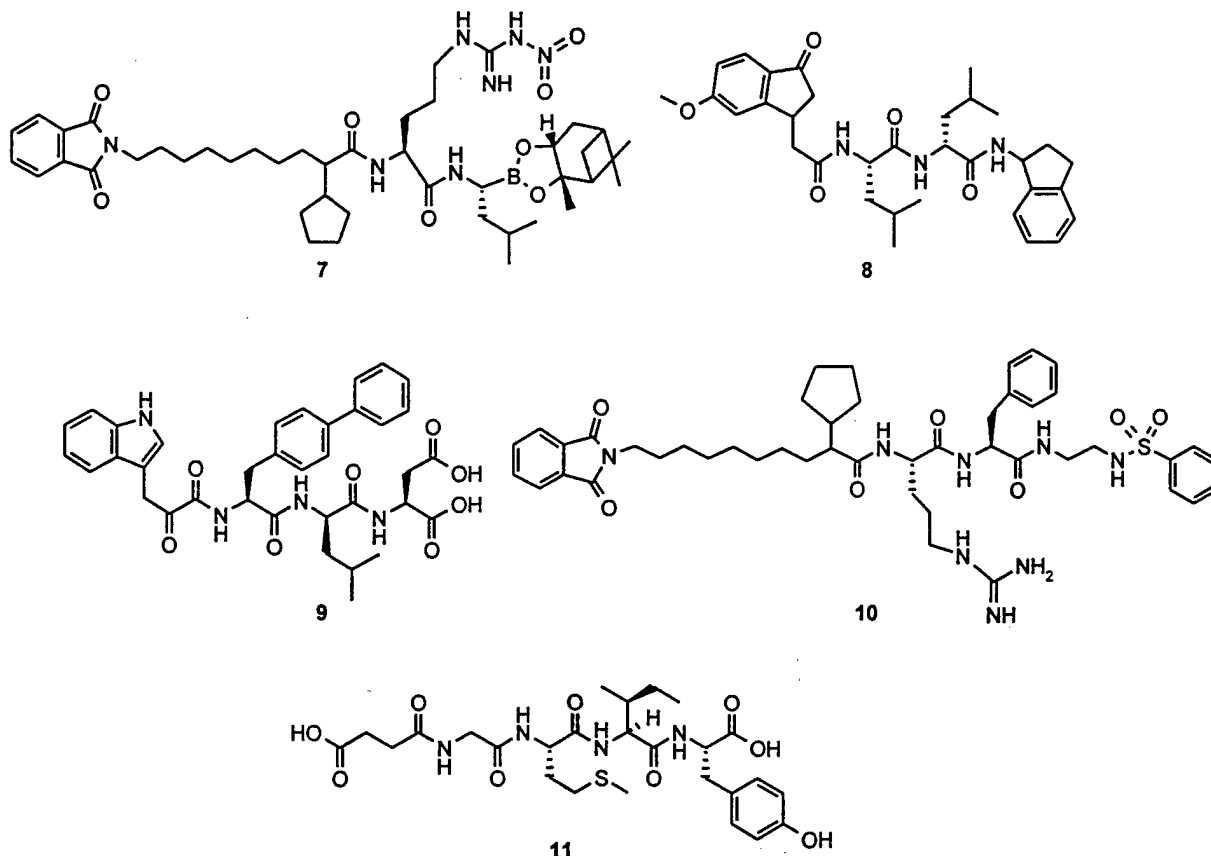
Therapeutics also disclosed 207 novel α-ketoamide derivatives, including **9**, as 20S proteasome inhibitors. These methods were further claimed for use in inhibiting cancer and treating autoimmune disorder diseases such as lupus erythematosus, multiple sclerosis, ARDS and arthritis [112]. Inhibition of the proteasomal chymotrypsin-like activity was measured using a proteasome complex purified from bovine brain. The IC<sub>50</sub> values ranged from 1 (**9**) to > 10 µg/ml in the applied *in vitro* assay. Cepharon also produced another series of novel α-ketoamide derivatives (39 compounds and intermediates) [113]. Compound **10**, which was modified from CEP1612 described in section 2.1, contains α-ketoamide moieties at the P' sites. The inventors claimed the use of these proteasome inhibitors for the treatment of muscle wasting disorders including muscular dystrophy, cardiac cachexia, emphysema, diabetes, leprosy, malnutrition, osteomalacia and cancer cachexia. It was also claimed for the treatment of the disorder characterised by reduction of superoxide dismutase-1 activity such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, Huntington's disease, stroke, trauma and ischaemia. The IC<sub>50</sub> values of the claimed 12 compounds were in a range of 2 - 139 nM under *in vitro* conditions. Compound **10** was also shown to be effective in reducing tumour volume in female mice with B16-F0 murine melanoma tumours at 10 mg/kg/day for 9 days.

### 3.7 Antitumour drugs

It has been found that several antitumour agents, such as cisplatin, 4'-(9-acridinyl-amino) methanesulphonm-anisidide (m-AMSA), mitomycin C and aclarubicin, inhibited the cell-free ubiquitin/proteasome-dependent proteolysis. However, IC<sub>50</sub> values of these antitumour agents were in a range of 50 - 300 µM [50,51]. In addition, the antitumour drug aclacinomycin A was found to selectively inhibit the chymotrypsin-like activity (IC<sub>50</sub> = 24 µM) of the proteasome over others. Aclacinomycin A had no effect on cathepsin B, stimulated trypsin and inhibited chymotrypsin, but had some effect on calpain [52]. It is possible that the proteasome inhibitory activity of these drugs contributes to their main antitumour activity.

### 3.8 Proteasome enhancer

In contrast to the above proteasome inhibitors, Sumitomo Electric claimed novel peptide compounds, their salts and agents possessed both 20S



proteasome-enhancing and immunopotentiating effects [114]. Only compound **11** was disclosed and its production described. The inventors also claimed the use of these compounds in cancer prevention and treatment. These compounds in the range of 0 - 30  $\mu$ M were shown to enhance the production of TNF- $\alpha$  by ~10-fold in cultured cells.

#### 4. Expert opinion

The involvement of the ubiquitin/proteasome-dependent degradation pathway in both upregulation of cell proliferation and downregulation of cell death in tumour cells suggests the potential use of proteasome inhibitors as novel anticancer drugs. This hypothesis has been supported by both *in vitro* and *in vivo* experimental results. *In vitro*, proteasome inhibitors rapidly induce apoptosis in a variety of human cancer cell lines, selectively trigger programmed cell death in the oncogene-transformed, but not normal or untransformed cells and are even able to activate the death program in human cancer cells that are resistant to various anticancer agents. *In vivo*, proteasome inhibitors suppress tumour growth *via* induction of

cancer cell apoptosis and inhibit the process of angiogenesis. In addition, results from Phase I studies of PS-341 demonstrates that this proteasome inhibitor has clinical activity without toxicity, further supporting the hypothesis that proteasome inhibitors can be used as therapeutic anticancer drugs. Implication of some important proteasome targets such as NF- $\kappa$ B in other human diseases suggests additional clinical potential of proteasome inhibitors.

Given the fact that the proteasome is involved in several important cellular processes, it will be a great challenge for researchers to design specific, selective and potent proteasome inhibitors. Ideally a proteasome inhibitor to be used for therapeutic purposes should not inhibit other protease activities, interact with other proteins and affect normal cells at therapeutic doses. It would be important that the proteasome inhibitor selectively accumulates only the pro-apoptotic or antiproliferative proteins (i.e., Bax, p53 and p27), but not the anti-apoptotic or pro-proliferative proteins (i.e., Bcl-2, MDM2 and cyclin E). Such a selectivity of the proteasome inhibitor should be associated with increased potency against tumour cells. Equally important,

understanding of the disease-related proteasome target proteins will accelerate the drug discovery process of novel proteasome inhibitors. It is to be hoped that more specific, selective and potent proteasome inhibitors will be developed in laboratories and used in clinics in the near future.

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